REGULATION OF THE NEURONAL-SPECIFIC TRYPTOPHAN HYDROXYLASE-2

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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30 September 2014

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SUMMARY

The dysregulation of the brain serotonergic system has been implicated in many neuropsychiatric disorders, including depression and schizophrenia, and thus is a common target for therapy. Tryptophan hydroxylases (TPH) are the rate-limiting enzymes that catalyze the biosynthesis of serotonin, which is the neurotransmitter involved in serotonergic signaling. TPH is present in the body as two isoforms. Tryptophan hydroxylase-1 (TPH1) is found mainly in peripheral tissues, while the more recently discovered tryptophan hydroxylase-2 (TPH2) is neuronal specific and controls brain serotonin synthesis. Initial efforts to characterize TPH2 have revealed that the enzyme is unstable *in vitro*, as compared to TPH1. However, the mechanisms involved in the regulation of TPH2 stability are yet to be elucidated. As such, the objective of this work is to examine the regulatory mechanisms that underlie the instability of TPH2 on a molecular level. In particular, we sought to identify novel regulatory partners that may be involved in the regulation of TPH2 stability. Furthermore, we investigated the role of the ubiquitin proteasome pathway in the degradation of TPH2, which may also affect its stability *in vitro*.

The present study has identified heat shock protein HSP70 as a novel protein interacting partner of both TPH1 and TPH2. HSP70 is observed to interact more strongly with TPH1 than with TPH2. We also

determined that interaction with HSP70 occurs through a non-conserved region of 10 amino acids within the N-terminal regulatory domains of TPH1 and TPH2. Functionally, our findings suggest that HSP70 can positively regulate TPH protein stability and expression. Furthermore, TPH2 is observed to be more sensitive to changes in levels of endogenous HSP70, as compared to TPH1, which indicated that the instability of TPH2 is partly due to its poor interaction with HSP70. We have also demonstrated, for the first time, that TPH2 can be ubiquitinated and degraded via the proteasome. Mutagenesis studies have also revealed that lysine-less TPH2 can still undergo ubiquitination, indicating that TPH2 may be ubiquitinated through alternative mechanisms.

Taken together, the results presented in this work have revealed previously undiscovered regulatory mechanisms that are involved in the regulation of TPH2 stability. These findings contribute to the understanding of the physiological regulation of the brain serotonergic system, which in turn can advance the development of new therapeutic interventions for neuropsychiatric disorders.

LISTS OF FIGURES AND TABLES

ABBREVIATIONS

2D-DIGE	2D-difference gel electrophoresis
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin, 5-hydroxytryptamine
5-HTP	5-Hydroxytryptophan
AADC	Aromatic L-amino acid decarboxylase
ADHD	Attention deficit hyperactivity disorder
BDM-PUB	Prediction of ubiquitination sites with Bayesian Discriminant Method
FLAG	DYKDDDDK polypeptide tag
HSP70	Heat shock protein 70 kDa
IP/ co-IP	Immunoprecipitation/ co-immunoprecipitation
KNK437	N-Formyl-3,4-methylenedioxy-benzylidine-γ-butyrolactam
MAO	Monoamine oxidase
MG132	N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide
PAH	Phenylalanine hydroxylase
PEST	Degradation motif, rich in proline, glutamic acid, serine, and threonine.
PKA	Protein kinase A
SERT	Serotonin transporter
SPR	Surface plasmon resonance
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
UBD	Ubiquitin binding domain

1. INTRODUCTION

Psychiatric disorders are one of the most pressing public health issues faced by the global population today. Indeed, the World Health Organization has reported that in 2004 mental disorders represent 13% of the global burden of disease, exceeding even cancer and cardiovascular diseases. In particular, depression, which affects 11% of the global population, alone accounted for 4.3% of the global burden of disease (Mathers, Fat et al. 2008; Collins, Patel et al. 2011). In 2012, about 18.6% of the adult population in the United States are affected by mental illnesses (SAMHSA 2013), while in Singapore, about 10% of the adult population are affected (Chong, Abdin et al. 2012). These debilitating conditions have a significant impact on both the global economy and individuals, and extensive research has been carried out over the years to develop treatments for these disorders.

The relationship between serotonergic signaling and neuropsychiatric disorders first emerged when monoamine oxidase inhibitors were shown to abolish the effects of depression (Bueno and Himwich 1967; Ban 2001). Over the years, the role of serotonin, in addition to the involvement of other monoamines such as dopamine and norepinephrine, in the pathophysiology of mental disorders has become more established (Ruhe, Mason et al. 2007). The serotonergic system, together with the other monoamine systems, is also a common target for many current treatments of neuropsychiatric conditions (Chen

and Skolnick 2007; Krishnan 2007). Nevertheless, the mechanisms by which serotonergic dysregulation can give rise to mental disorders are still not fully understood. As such, much effort has been placed into studying the workings of the serotonergic system, on both systemic and molecular levels, which in turn can also provide new insights into the development of new therapeutic drugs in the treatment of these disorders.

1.1 THE BRAIN SEROTONERGIC SYSTEM

The serotonergic system in the human body mainly regulates physiological functions in the gastrointestinal tract, blood, and central nervous system (CNS). Signaling within the system is carried out by the monoamine neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), which in turn gives rise to changes in physiological processes. As the serotonergic system of the CNS has been implicated to play an imperative role in the pathophysiology of neuropsychiatric disorders, the focus of this work will be on the brain serotonergic system rather the peripheral serotonergic system.

1.1.1 Structure of the brain serotonergic system

In order to better understand its functions, the structure of the brain serotonergic system will be first elaborated. The actions of the brain serotonergic system are carried out by an intricate network, which

includes 5-HT synthesis, 5-HT receptor signaling, and 5-HT reuptake by transporters (Figure 1.1, A and B).

1.1.1.1 Serotonergic neurons within the brain

The cell bodies of serotonergic neurons are found on or near the midline of the brain stem, organized in clusters known as the raphe nuclei. They can be further separated into two groups, according to their projections within the brain (Figure 1.1B). The superior or rostral group mainly comprises of the caudal linear nucleus, median raphe nucleus, dorsal raphe nucleus, and B9 neurons dorsal of the medial lemniscus. On the other hand, the inferior or caudal group is made up of the nucleus raphe obscurus, nucleus raphe pallidus, nucleus raphe magnus and neurons from the lateral reticular formation. Raphe nuclei in the rostral group receive afferent inputs primarily from the limbic system within the forebrain. In turn, serotonergic fibers originating from the rostral group project to brain areas that perform higher function, such as the forebrain, cerebral cortex, hippocampus, hypothalamus, and thalamus. Afferent inputs to the caudal group can originate from the hypothalamus, thalamus, amygdala and the reticular formation of the medulla. Consequently, serotonergic projections from the caudal group can innervate the brain stem and spinal cord (Dahlström and Fuxe 1964; Jacobs and Azmitia 1992; Hornung 2003).

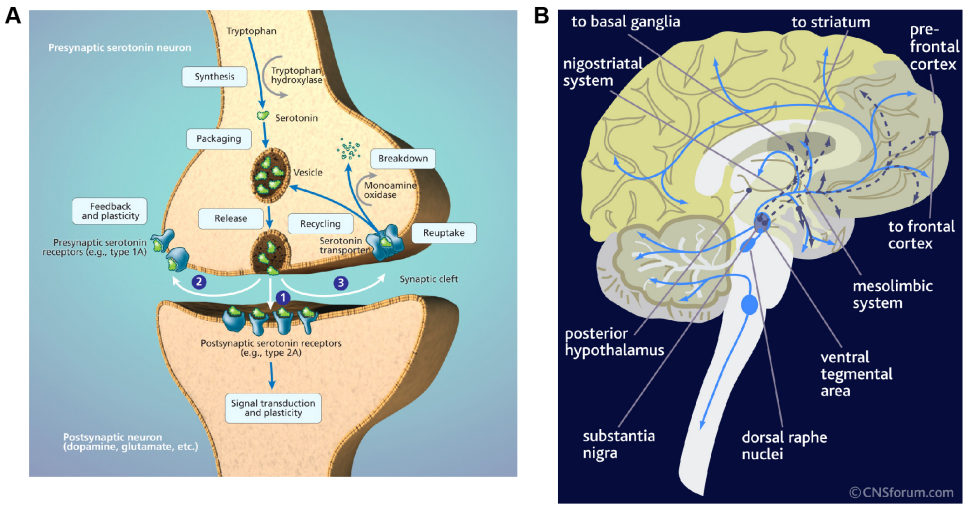

FIGURE 1.1. The brain serotonergic system. A, The 5-HT network at the synapse. 5-HT is synthesized by tryptophan hydroxylase within the serotonergic neurons and stored in vesicles, before being released into the synaptic cleft. Extracellular 5-HT can then associate with pre- or post-synaptic 5-HT receptors to bring about physiological changes (1 and 2). 5-HT reuptake is mediated by the serotonin transporter, which transports 5-HT back into the serotonergic neurons, where it is broken down by monoamine oxidase (3). (Image from (aan het Rot, Mathew et al. 2009)). B, Serotonergic projections from the raphe nuclei in the brain stem are highlighted in light blue. Efferent projections from the rostral group, including the dorsal raphe nuclei, innervate regions of the brain performing higher function, while serotonergic fibers from the caudal group project to the brain stem and spinal cord. (Image from CNSforum, The Lundbeck Institute)

1.1.1.2 Serotonin receptors

Serotonergic neurotransmission is mediated by 5-HT receptors, which are activated when bound to 5-HT released from serotonergic neurons. This in turn leads to the activation of other downstream signaling cascades. To date, there are seven known families of 5-HT receptors, with a total of fourteen subtypes, which have specific pre- and post-synaptic localizations. While the 5-HT_3 receptor is a ligand-gated ion channel, most 5-HT receptors are members of the seven transmembrane G-protein coupled receptor family. As such, they can bring about excitatory or inhibitory responses, depending on the coupled G-protein (Nichols and Nichols 2008). Given their essential roles in the brain serotonergic system, 5-HT receptors can influence a wide range of biological and behavioral processes. Thus, 5-HT receptors are potential targets for therapeutic drugs, such as antidepressants and antipsychotics (Glennon 1987; Nichols and Nichols 2008; Meltzer, Massey et al. 2012).

1.1.1.3 Termination of serotonergic actions

5-HT released into the synapse is removed by a specialized serotonin transporter protein (SERT), which is a 12 transmembrane, sodium- and chloride-dependent transporter, resulting in the termination of serotonergic action. As the amount of extracellular 5-HT within the synaptic cleft can influence 5-HT signaling, 5-HT reuptake by SERT can also modulate 5-HT neurotransmission and bring about

changes in behavior or homeostasis. It is on this basis that SERT has also become a pharmacological target for many selective serotonin reuptake inhibitors (SSRIs), which exert their effects by modulating the amount of extracellular 5-HT present (White, Walline et al. 2005; Nichols and Nichols 2008).

1.1.2 Functions of the brain serotonergic system

In the brain, the serotonergic system is predominantly involved in the modulation of higher order behavioral functions, such as mood, sleep, circadian rhythm, appetite, aggression, sexual behavior, as well as cognitive functions such as learning and memory (Simansky 1996; Lucki 1998; Canli and Lesch 2007). The dysregulation of the brain serotonergic system may lead to the disruption of these functions. Indeed, pharmacological intervention of serotonergic regulation has been indicated to alleviate symptoms of many neuropsychiatric disorders, such as depression, anxiety, bipolar disorder and schizophrenia (Coppen 1967; Duman, Heninger et al. 1997; Mann 1999; Meltzer, Li et al. 2003). In addition, disruptions in 5-HT synthesis, as well as 5-HT receptors and SERT functions, have also been associated with these psychiatric conditions (Hoyer, Hannon et al. 2002; Murphy, Uhl et al. 2003; Matthes, Mosienko et al. 2010).

In addition to its role in regulating behavior, it is becoming increasingly evident that the brain serotonergic system is also involved

in adult neurogenesis within the hippocampus (Gould 1999; Brezun and Daszuta 2000; Banasr, Hery et al. 2003; Alenina and Klempin 2014). This generation of new hippocampal neurons may be important for learning and memory. Interestingly, treatment with SSRIs has been shown to improve neurogenesis and synaptic plasticity (Malberg, Eisch et al. 2000; Wang, David et al. 2008; Chen, Pan et al. 2014). Furthermore, several findings have also suggested that exercise can induce neurogenesis, and this process may be mediated by the action of 5-HT (van Praag, Kempermann et al. 1999; Kronenberg, Reuter et al. 2003; Klempin, Beis et al. 2013).

1.2 SEROTONIN

Central to the serotonergic system is serotonin (5-hydroxytryptamine, 5-HT), a major monoamine neurotransmitter and hormone. Originally discovered in blood serum and gastric mucosa as an inducer of vasoconstriction and gastrointestinal contraction (Rapport, Green et al. 1948; Erspamer and Asero 1952), 5-HT is now known to play a role in a wide range of physiological functions. In order to advance the development of treatments for conditions in which the serotonergic system is disrupted, an understanding of the biosynthetic pathway of 5-HT and regulatory mechanisms involved is necessary.

1.2.1 Biosynthesis and metabolism

5-HT is synthesized by a two-step biochemical pathway (Tyce 1990) (Figure 1.2). L-tryptophan is first converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). This TPH-mediated reaction is also the rate-limiting step in the synthesis of 5-HT. In the second step of the process, 5-HTP is converted by L-aromatic amino acid decarboxylase to form 5-HT in a decarboxylation reaction. Subsequently, 5-HT can be broken down by oxidative deamination to produce 5-hydroxyindoleacetic acid (5-HIAA), in a reaction catalyzed by monoamine oxidases (MAO), particularly MAO-A.

1.2.2 Tryptophan hydroxylase

Tryptophan hydroxylase (TPH) belong to a family of aromatic amino acid hydroxylases, together with tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH) (Figure 1.3). Similar to the other two enzymes, TPH consists of an N-terminal regulatory domain, a central catalytic domain, and a C-terminal tetramerization domain (Fitzpatrick 1999). The presence of its C-terminal domain allows TPH to exist under physiological conditions as a homotetramer, which is required for the functionality of the enzyme. In addition, TPH is a tetrahydrobiopterin (BH_4)-dependent enzyme. As such, TPH also requires the BH_4 cofactor and iron to bring about enzymatic activity (Fitzpatrick 2003). In members of the aromatic amino acid hydroxylase

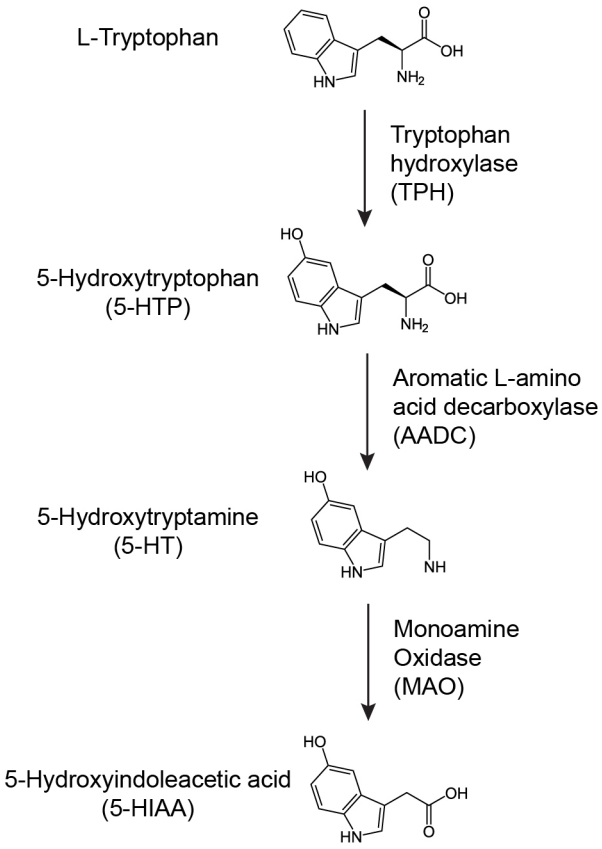

FIGURE 1.2. Serotonin synthesis pathway. TPH converts L-tryptophan into 5-HTP in the first, rate-limiting step. 5-HTP is then decarboxylated by AADC to form 5-HT in the second step. 5-HT can be broken down by MAO to form 5-HIAA, which is excreted in urine.

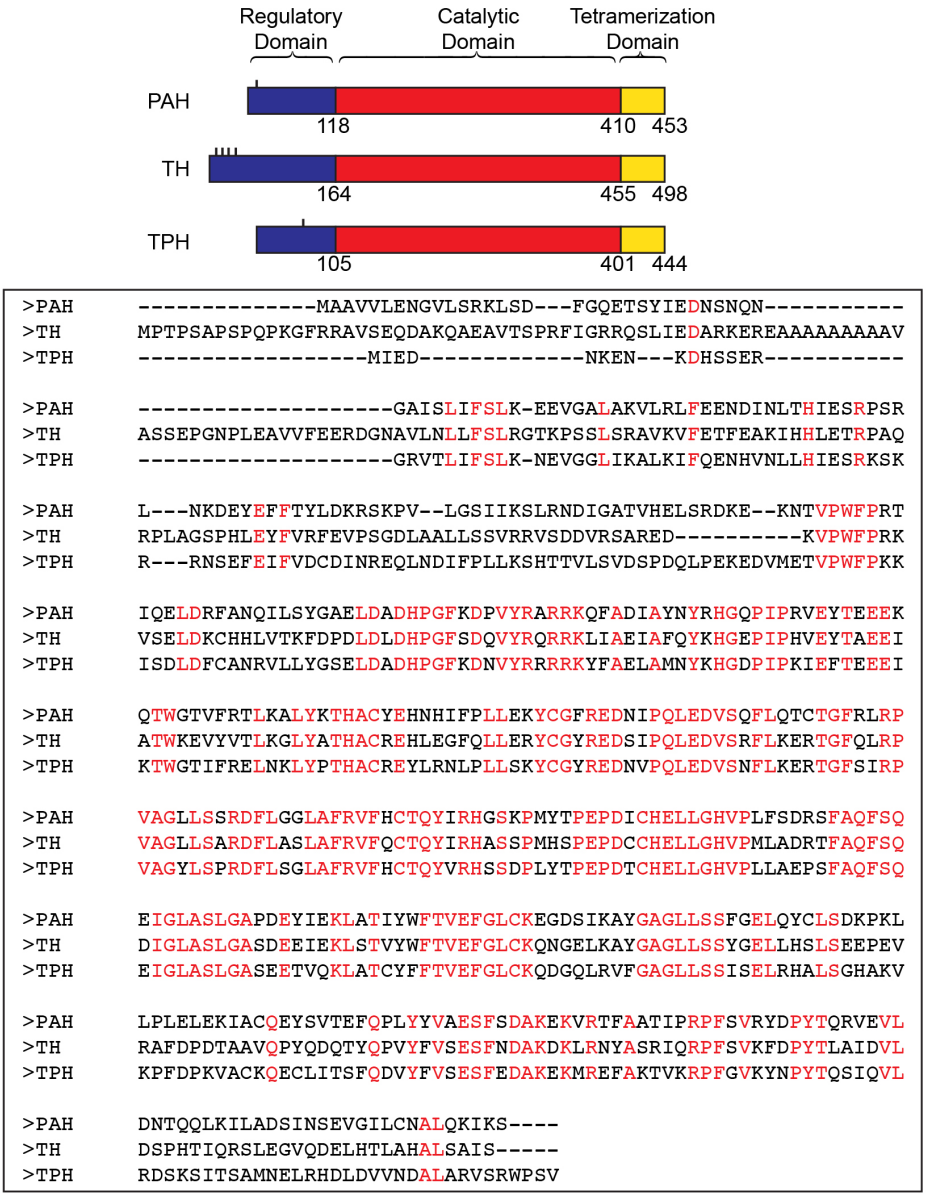

FIGURE 1.3. Aromatic amino acid hydroxylases. Domain structures and sequences of rat PAH, TH and TPH are compared. Regulatory domains are denoted in blue, catalytic domains in red, and tetramerization domains in yellow. Phosphorylation sites within the regulatory domains, which play a role in differential regulation of protein expression and activity, are represented as vertical lines (Adapted from (Fitzpatrick 1999)). ClustalW sequence alignment of the amino acid sequences of rat PAH, TH and TPH revealed that the N-terminal regulatory domains are divergent in length and sequence, while the catalytic and tetramerization domains are more homologous. Conserved amino acids are highlighted in red.

family, amino acid sequences within the catalytic and C-terminal domains are relatively homologous. The C-terminal domains also form conserved hydrophobic coiled-coil motifs, or leucine zippers, that facilitate the formation of homotetramers (Liu and Vrana 1991; Vrana, Walker et al. 1994; Knappskog, Flatmark et al. 1996; Carkaci-Salli, Flanagan et al. 2006). However, their regulatory domains share very little similarities, in both sequence and structure, which may give rise to differences in substrate specificities as well as unique regulatory characteristics (Figure 1.3). This dissimilarity is also evident between the two isoforms of TPH.

1.2.2.1 Tryptophan hydroxylase 1

Tryptophan hydroxylase 1 (TPH1) was first described and characterized in the 1960s (Grahame-Smith 1964; Lovenberg, Jequier et al. 1967; Jequier, Robinson et al. 1969). The gene consists of 11 exons, and was mapped to chromosome 11 and chromosome 7 in human and mouse, respectively (Ledley, Grenett et al. 1987; Stoll, Kozak et al. 1990; Stoll and Goldman 1991; Nielsen, Dean et al. 1992). TPH1 is mainly expressed in peripheral tissues, namely intestinal enterochromaffin cells, blood platelets, beta cells of the islets of Langerhans, as well as the pineal gland (Côté, Thévenot et al. 2003; Kim, Toyofuku et al. 2010). Nevertheless, TPH1 is also detected in neuronal tissues, albeit in low levels (Patel, Pontrello et al. 2004; Gundlah, Alves et al. 2005; Malek, Dardente et al. 2005; Zill, Buttner et

al. 2007). About 95% of the 5-HT in the body is present in the periphery, particularly in the gastrointestinal tract, where it serves to regulate peristaltic movements and intestinal secretion. 5-HT can also be stored in platelets, where it regulates vascular tone. Peripheral 5-HT has also been indicated to play a role in cardiac morphorgenesis during prenatal development (Yavarone, Shuey et al. 1993). Within the pineal gland, 5-HT acts as an intermediate in the biosynthesis of melatonin, which in turn is involved in the control of circadian rhythm (Reiter 1991).

1.2.2.2 Tryptophan hydroxylase 2

TPH has long been considered to exist as a single isoform and extensively studied as such. However, when TPH1 was ablated in mice, levels of 5-HT within the brain was observed to remain relatively unchanged. This lead to the identification of a second isoform of TPH, tryptophan hydroxylase 2 (TPH2) (Walther and Bader 2003; Walther, Peter et al. 2003). The TPH2 gene comprises of 11 exons and has been mapped to chromosome 12 in human, and chromosome 10 in mouse. TPH2 expression is primarily limited to the central nervous system (CNS), mainly the neurons of raphe nuclei within the brain stem (Côté, Thévenot et al. 2003; Walther, Peter et al. 2003; Patel, Pontrello et al. 2004). In humans and mice, expression of TPH2 has also been detected in the cortex, hippocampus and cerebellum (Zill, Buttner et al. 2007; Gutknecht, Kriegebaum et al. 2009). As a neuronal specific

isoform, TPH2 controls 5-HT synthesis within the brain (Zhang, Beaulieu et al. 2004), which in turn plays a role in the regulation of behavioral functions. These include mood, stress, cognition, synaptic plasticity, sleep and even sexual behavior (Lucki 1998; Gutknecht, Kriegebaum et al. 2009). Although the expression of TPH1 and TPH2 were thought to be mutually exclusive, subsequent studies also detected the expression of TPH2 in several peripheral sites in rodents, such as the enteric neurons in the gut, bile duct, islets of Langerhans, and adipose tissues (Côté, Thévenot et al. 2003; Hageman, Wagener et al. 2010; Schraenen, Lemaire et al. 2010; Omenetti, Yang et al. 2011), suggesting that TPH2 may also control 5-HT signaling in the periphery, possibly as part of the stress response (Chen and Miller 2013).

1.2.2.3 Insights to TPH1 and TPH2 function from in vivo models

Studies performed using transgenic models have also further contributed to the understanding of functions that are controlled by TPH1 and TPH2 on a systemic level.

Tph1 knockout mice have reduced peripheral 5-HT levels, and exhibit palor, fatigue and difficulties in breathing. More importantly, these Tph1$^{-/-}$ mice display functional cardiac abnormalities that can progressively lead to heart failure (Côté, Thévenot et al. 2003). A separate study has also shown that Tph1$^{-/-}$ mice are diabetic and have

disrupted insulin secretion as a result of the absence of 5-HT in the pancreas (Paulmann, Grohmann et al. 2009). Moreover, liver regeneration was observed to be impaired in partially hepatectomized $Tph1^{-/-}$ mice (Lesurtel, Graf et al. 2006). In addition, mice that are deficient in Tph1 in the gut ($Tph1_{gut}^{-/-}$) exhibit an increase in osteoblast proliferation and bone formation, resulting in severely high bone mass (Yadav, Ryu et al. 2008). However, this increase in bone mass is not observed in global knockout $Tph1^{-/-}$ mice, suggesting the possible involvement of compensatory pathways (Cui, Niziolek et al. 2011).

Tph1 has also been demonstrated to play a crucial role in development. Both heterozygous ($Tph1^{+/-}$) and homozygous ($Tph1^{-/-}$) offsprings of $Tph1^{-/-}$ mice are smaller in size, and display abnormalities in morphology, particularly in the brain. This in turn indicated that maternal 5-HT may play morphogen-like role in embryonic development (Côté, Fligny et al. 2007). Furthermore, Tph1 is shown to be expressed in murine raphe at postnatal day 22, and thus may also be involved in the synthesis of 5-HT during the late developmental stages of the brain (Nakamura, Sugawara et al. 2006). Taken together, these studies in $Tph1^{-/-}$ mice have indicated that peripheral 5-HT is involved in development, liver regeneration, regulating insulin secretion, as well as controlling bone mass.

The functions of Tph2 have been studied *in vivo* using inbred mouse strains with different allelic distributions of the SNP C1473G, which encodes for a missense mutation P447R in Tph2 that causes a reduction in its activity (Zhang, Beaulieu et al. 2004). The allelic differences have been demonstrated to give rise to behavioral changes, with mice homozygous for the 1473G allele displaying altered responses when tested for anxiety, aggression and depressive-like behaviors. This suggests that changes in the Tph2 activity can affect behavior (Lucki, Dalvi et al. 2001; Osipova, Kulikov et al. 2009; Berger, Weber et al. 2012). A separate study also demonstrated that the different allelic distributions could result in differential reponses to SSRI treatment. This supports the role of TPH2 in regulating brain 5-HT homeostasis, and also indicates its involvement in resistance to SSRI treatment (Cervo, Canetta et al. 2005).

In addition, knock-in mice that express the R439H Tph2 allele (R439H Tph2 KI) and Tph2 knockout $Tph2^{-/-}$ mice have also been studied. The R439H Tph2 allele is analogous to a rare human R441H Tph2 variant, encoded by the SNP G1463A. This SNP has been identified in patients with major depression, and causes an 80% decrease in Tph2 expression and 5-HT synthesis (Zhang, Gainetdinov et al. 2005). R439H Tph2 KI mice display aberrant behaviors, such as increased anxiety, aggression, and depressive-like symptoms, further supporting the role of Tph2 in modulating behavior (Beaulieu, Zhang et

al. 2008). Chronic treatment of R439H Tph2 KI mice with SSRI antidepressants, like fluoxetine and paroxetine, also resulted in an exacerbation of brain 5-HT deficiency. Nevertheless, this effect can be restored by co-treatment with 5-HTP, indicating that Tph2 is involved in the proper maintaining of brain 5-HT levels during chronic SSRI treatment (Siesser, Sachs et al. 2013).

Unlike R439H Tph2 KI mice, where 5-HT synthesis is reduced, Tph2 is ablated in $Tph2^{-/-}$ mice. While $Tph2^{-/-}$ mice can survive into adulthood, a lethality rate of 50% is observed within the first four weeks. In addition, the absence of brain 5-HT signaling resulted in retarded growth, as well as alterations in sleep pattern, thermoregulation, cardiovascular functions and breathing. Maternal behavior of $Tph2^{-/-}$ mice is also affected, leading to poor care and survival of offsprings (Alenina, Kikic et al. 2009). Collectively, studies in Tph2 mutant mice have shown that Tph2 plays a role during development, as well as in behavioral modulation. In addition, disruption of Tph2 is also indicated to give rise to abnormalities in behavior, thus implicating its role in neuropsychiatric disorders.

1.2.3 Similarities and differences in properties of the two TPH isoforms

TPH1 and TPH2 share about 70% identity in their protein sequences, with their catalytic and tetramerization domains being

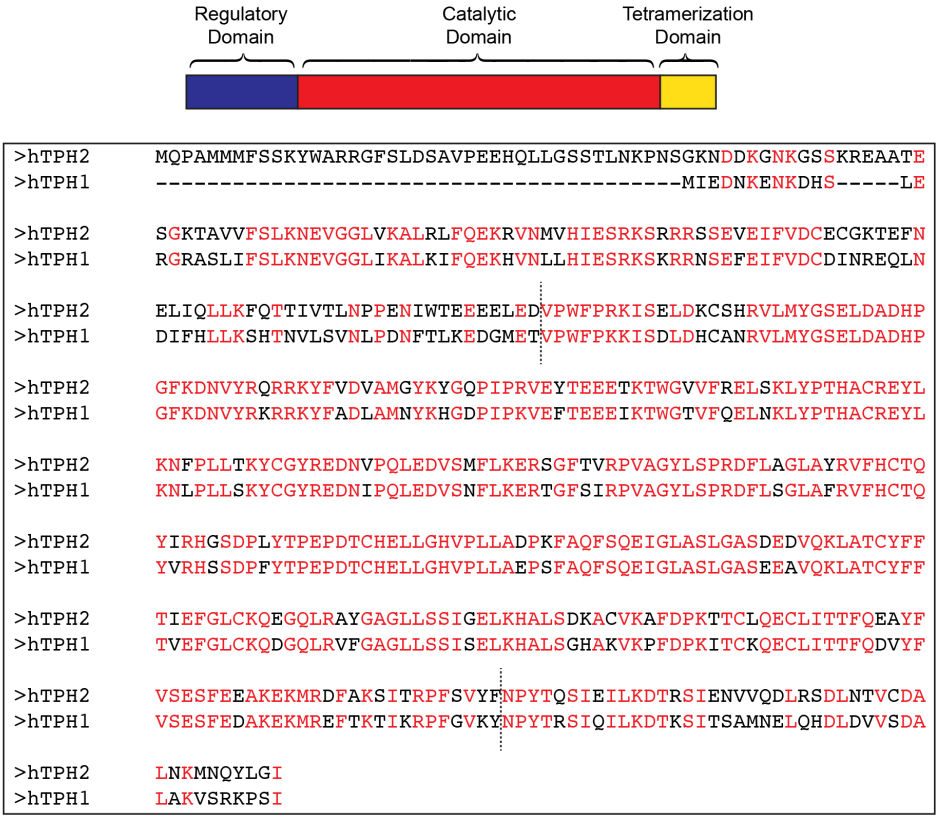

FIGURE 1.4. Comparison of amino acid sequences of human TPH1 (hTPH1) and human TPH2 (hTPH2). Sequence alignment performed using ClustalW revealed that the catalytic and tetramerization domains of TPH1 and TPH2 have considerable homology. However, their N-terminal regulatory domains are not conserved. In particular, the first 58 amino acids are unique to TPH2. Furthermore, the first 41 amino acids of TPH2 are not present in the TPH1. The regulatory N-terminus of TPH2 comprises of the first 150 amino acids. Dashed lines denote domain boundaries. Conserved amino acids are represented in red.

highly homologous (Figure 1.4) (Walther and Bader 2003). Furthermore, residues that play crucial roles in the structure and function of TPH1 are conserved in TPH2 (Jiang, Yohrling et al. 2000; Martinez, Knappskog et al. 2001; McKinney, Teigen et al. 2001; Daubner, Moran et al. 2002; Wang, Erlandsen et al. 2002). Both TPH1 and TPH2 can also be phosphorylated at specific serine residues (Jiang, Yohrling et al. 2000; McKinney, Knappskog et al. 2005; Kuhn, Sakowski et al. 2007; Winge, McKinney et al. 2008). Despite these similarities, characterization studies have revealed numerous differences that clearly distinguish the two TPH isoforms.

In addition to the differences in spatial expression (Sakowski, Geddes et al. 2006), amino acid sequences within the regulatory domains of both TPH isoforms were strikingly divergent. TPH2 has an extended regulatory domain as compared to that of TPH1, and the first 58 amino acids of TPH2 are unique to the isoform, with 41 amino acids not present in TPH1 (Figure 1.4) (Walther and Bader 2003). This difference in length at the N-terminal is reflected in the protein sizes, with each TPH2 subunit (57 kDa) having a slightly higher molecular weight as compared to TPH1 (51 kDa). Kinetic characterization on enzyme activity in bacteria has also revealed that TPH1 has higher V_{max} and K_m values for BH_4 than TPH2, although TPH2 has a higher K_m value for tryptophan as compared to TPH1. This indicates that TPH2 has a higher substrate preference and binding affinity to tryptophan,

but a lower maximum activity, as compared to TPH1, (McKinney, Knappskog et al. 2005). More importantly, physiological studies have demonstrated that TPH2 exhibits lower stability as compared to TPH1 when expressed in mammalian cells, and is also less efficiently synthesized (Murphy, Zhang et al. 2008). As the focus of this present work is on the regulation of TPH2, TPH1 will only be mentioned as a comparison, but not further discussed in the subsequent sections of this chapter.

1.2.4 Role of the TPH2 regulatory domain

Given its distinct amino acid sequence in the regulatory domain, the extended N-terminal of TPH2 is the subject of interest in several studies as it may confer regulatory mechanisms that are unique to TPH2. This in turn can result in the differential properties that distinguish TPH2 from TPH1. *In vitro* studies using mammalian and bacterial cells have been carried out to characterize the role of the TPH2 N-terminal regulatory domain with respect to its protein expression.

The TPH2 protein is generally insoluble and thus difficult to purify in its native form (McKinney, Knappskog et al. 2005). However, by removing the regulatory domain of human TPH2, enzyme solubility was significantly increased. In addition, deletion of the regulatory domain also increased TPH2 stability by approximately six-fold

(Carkaci-Salli, Flanagan et al. 2006). A separate study carried out in mammalian cells reported that protein expression level of TPH2 was lower compared to TPH1, even when mRNA levels were not altered. *In vitro* translation and cycloheximide treatment also showed that TPH2 was synthesized at a slower rate, but underwent degradation faster than TPH1. Furthermore, these negative effects on TPH2 expression was found to be brought about by the first 58 amino acids of the extended N-terminus of TPH2, with residues 11-20 contributing to the bulk of the effect (Murphy, Zhang et al. 2008).

As such, the findings suggest that the N-terminal regulatory domain can negatively regulate TPH2 activity through reduction of protein synthesis and expression, decreasing enzyme solubility and destabilization. However, the underlying mechanisms by which the TPH2 regulatory domain gives rise to these effects are still unknown.

1.3 REGULATION OF TRYTOPHAN HYDROXYLASE 2

Since the discovery of the neuronal specific TPH2, many studies have been done to further understand the regulation of the activity and expression of this enzyme in the brain. These lines of research not only provide new insights to the processes that are involved in brain 5-HT synthesis, but also contribute to development of new therapeutic strategies that can improve brain 5-HT production as well. It is now known that TPH2 expression within the brain can be regulated on both

genetic and protein levels, and current knowledge will be discussed in this section. However, as this work will focus on TPH2 protein regulation in the later chapters, more emphasis will be given to regulatory mechanisms at the protein level in this section.

1.3.1 Gene regulation and external factors

The regulation of gene expression is the process by which cells control the production of gene products, and is usually dependent on the untranslated regions of the gene. An early study investigating TPH2 polymorphisms in rhesus monkeys has suggested that the 3' untranslated region (3'UTR) can affect the stability of the mRNA transcript and translation efficiency (Chen, Novak et al. 2006). Characterization of the 5' untranslated region (5'UTR) of human TPH2 has also revealed that the 5'UTR exerts an inhibitory effect on TPH2 gene expression (Chen, Vallender et al. 2008), which may be species specific (unpublished data). Furthermore, later studies identified an asymmetrical bidirectional promoter and an estrogen response element half-site within the 5'UTR of TPH2, suggesting that TPH2 expression can be regulated by non-coding RNAs as well as estrogen receptor β and its agonists (Chen and Miller 2009; Hiroi and Handa 2013). Transcription factors, such as POU3F2 (N-Oct-3) and RE-1 silencer of transcription (REST/NRSF), have also been revealed to associate with the TPH2 5'UTR and can regulate TPH2 gene expression by activating or repressing transcription, respectively (Patel, Bochar et al. 2007;

Scheuch, Lautenschlager et al. 2007). In addition to gene regulation at the 5' and 3' UTRs, TPH2 mRNA has been demonstrated to undergo alternative splicing and RNA editing, indicating the role of post-transcriptional regulation in TPH2 expression as well (Grohmann, Hammer et al. 2010).

Changes in TPH2 gene expression can be influenced by external factors. Several studies have illustrated the fluctuation of TPH2 expression according to a circadian rhythm (Liang, Wessel et al. 2004; Malek, Dardente et al. 2005; Nexon, Sage et al. 2011). Furthermore, hormonal levels, estrogen in particular, have been shown to regulate TPH2 mRNA transcript levels (Hiroi, McDevitt et al. 2006; Donner and Handa 2009; Bethea, Smith et al. 2011; Charoenphandhu, Teerapornpuntakit et al. 2011; Hiroi and Handa 2013). Interestingly, diet has also been implicated in TPH2 mRNA expression (McNamara, Able et al. 2009; Hageman, Wagener et al. 2010; Sullivan, Grayson et al. 2010). Finally, there have been increasing evidence indicating that TPH2 gene expression can be regulated by stress and glucocorticoids (Brown, Henderson et al. 2006; Malek, Sage et al. 2007; McEuen, Beck et al. 2008; Gardner, Hale et al. 2009; Heydendael and Jacobson 2009; Chen and Miller 2012; Donner, Montoya et al. 2012). Nevertheless, with the exception of estrogen regulation, molecular mechanisms underlying the crosstalk between the environmental triggers and TPH2 gene expression have not been fully elucidated.

1.3.2 Post-translational modifications

Post-translational modifications occur after proteins are synthesized, and act to control the functions and alter the structures of the proteins through the attachment of biochemical functional groups. Characterization studies on TPH1 have suggested that post-translational phosphorylation by protein kinases are involved in their regulation and thermal stability (Jiang, Yohrling et al. 2000). As TPH2 share 70% homology with TPH1, it is likely that TPH2 can also be modulated by phosphorylation. Incidentally, even before the identification of TPH2, TPH isolated from the brain stem has already been demonstrated to be phosphorylated and activated by protein kinases, namely protein kinase A (PKA) and calcium, calmodulin-dependent kinase II (CaMKII) (Ehret, Cash et al. 1989; Johansen, Jennings et al. 1996). These early observations were further confirmed in later studies, after TPH2 was recognized as a neuronal specific TPH isoform.

Using mass spectrometry analyses, separate studies have shown that phosphorylation of TPH2 can occur on serine residue 19 (S19). This modification can be mediated by both PKA as well as CaMKII, which in turn can increase the catalytic activity of the enzyme two fold (McKinney, Knappskog et al. 2005; Kuhn, Sakowski et al. 2007). Another study has also demonstrated that the induction of PKA by forskolin can result in the phosphorylation of S19 in wild type mouse

TPH2, as well as bring about an increase in protein expression and 5-HT synthesis. Using site-directed mutagenesis, the study also showed that the increase in TPH2 expression and activity was reduced in a S19A mutant, whereas a pseudophosphorylated S19D mutant of TPH2 exhibited a 2.5 fold increase in protein and 5-HT production (Murphy, Zhang et al. 2008). In addition to S19, both PKA and CaMKII have been shown to phosphorylate serine residue S104, which also leads to increased TPH2 activity and stability. Furthermore, phosphorylation at either S19 or S104 was found to increase the rate of phosphorylation on other phosphorylation sites in the enzyme, indicating that different kinase pathways may regulate TPH2 synergistically (Winge, McKinney et al. 2008). Taken together, these findings supported the earlier observations and further established that TPH2 protein expression and activity can be regulated by phosphorylation.

In addition to the role of phosphorylation in TPH2 regulation, findings from recent studies have suggested that other post-translational modifications may also occur on TPH2. Firstly, lysine residue 79 (K79) has been identified as a potential acetylation site, as detected by tandem liquid chromatography tandem mass spectrometry (LC/LC-MS/MS) in total liver protein lysates (Zhao, Xu et al. 2010). However, the detection of TPH2 in liver tissues is unexpected, and there have yet to be any reports validating the acetylation on K79. Nevertheless, protein acetylation can play a role in enzyme activation

or inhibition, as well as regulating protein stability (Zhao, Xu et al. 2010). As such, it would be interesting to determine whether K79 can indeed be acetylated, and how acetylation may affect the regulation of TPH2 expression and activity. Secondly, a separate study has demonstrated that all thirteen cysteine residues on TPH2 are able to undergo disulphide cross-linking in the presence of oxidative stressors. As this modification does not exhibit residue specificity, any cysteine residue on TPH2 can be involved in the cross-linking reaction. Functionally, disulphide cross-linking was found to cause the formation of TPH2 aggregates *in vitro*, resulting in inhibition of TPH2 activity (Kuhn, Sykes et al. 2011). Collectively, studies have demonstrated that phosphorylation, formation of disulphide linkages and possibly acetylation can occur on TPH2. Nevertheless, it is still uncertain whether TPH2 can be modified by other post-translational modifications.

1.3.3 Protein-protein interactions

In addition to biochemical modifications, interactions with other protein partners can also play a role in regulating protein function and activity. This is evident in most essential cellular processes, such as DNA replication, translation and signaling transduction. As such, identifying protein-protein interactions involving TPH2 can contribute to a better understanding of its regulatory network, which in turn may

allow us to uncover novel targets in the treatment of conditions involving dysregulation of the brain serotonergic system.

To date, 14-3-3 proteins are the only known interacting partners of TPH2. Prior to the recognition of TPH2 as a separate isoform from TPH1, TPH isolated from rabbit brain has already been demonstrated to bind to 14-3-3 proteins, under conditions where TPH was phosphorylated. Complex formation between phosphorylated TPH and 14-3-3 proteins is also shown to activate TPH and increase enzyme activity, and at the same time prevent TPH dephosphorylation (Banik, Wang et al. 1997). A later study further supported these observations by showing that PKA phosphorylation of human TPH2 can result in high affinity binding to some 14-3-3 proteins, such as 14-3-3γ, 14-3-3ε and BMH1. This interaction with 14-3-3 proteins further increases the catalytic activity and stability of TPH2 (Winge, McKinney et al. 2008). Nevertheless, the site of interaction between TPH2 and the 14-3-3 proteins, as well as the mechanisms by which 14-3-3 proteins stabilize TPH2, have not been fully elucidated.

1.4 IMPLICATIONS OF TPH2 IN PSYCHIATRIC DISORDERS

With the recognition of TPH2 as the neuronal specific, rate-limiting enzyme in the synthesis of brain 5-HT, many association and polymorphism studies, on both genetic and protein levels, have implicated TPH2 in a wide range of neuropsychiatric disorders, where

regulation of the serotonergic system is disrupted. Although by no means exhaustive, some association studies focusing on single nucleotide polymorphisms (SNP) in humans will be highlighted in this section, illustrating the role of TPH2 dysregulation in neuropsychiatric conditions.

1.4.1 Affective disorders

Affective disorders are a group of disorders in which mood is affected, and are made up of several psychiatric conditions. Major depressive disorder, or unipolar depression, and bipolar disorders are the most common. As affective disorders are complex disorders, it is possible that a single SNP can give rise to several conditions (Lesch 2004).

1.4.1.1 Major depressive disorder

Major depression is a disabling condition whereby mood, sleep, appetite, cognition and psychomotor activity may be affected (Fava and Kendler 2000). Many studies have already associated different SNPs within the TPH2 gene to major depression (Zill, Baghai et al. 2004; Zhang, Gainetdinov et al. 2005; Zhou, Roy et al. 2005; Van Den Bogaert, Sleegers et al. 2006; Tsai, Hong et al. 2009; Gao, Pan et al. 2012). Although most SNPs are located in introns and untranslated regions, two rare SNPs within the TPH2 coding region have been detected. Notably, the SNP G1463A encodes a missense mutation

(R441H) in the TPH2 protein, resulting in a significant decrease in 5-HT synthesis (Zhang, Gainetdinov et al. 2005). However, the rarity of this functional polymorphism has resulted in varying reports of its presence by various groups (Garriock, Allen et al. 2005; Zhou, Roy et al. 2005). In addition, the SNP rs17110563 causes a P206S substitution in TPH2, resulting in reduced enzyme stability and solubility (Zhou, Roy et al. 2005; Cichon, Winge et al. 2008). Taken together, detection of SNPs within the TPH2 gene in depressed patients suggests that TPH2 is associated with major depression.

1.4.1.2 Bipolar disorder

Bipolar affective disorder is a mental condition whereby patients can experience recurrent fluctuations of elevated mood and depression (Anderson, Haddad et al. 2012). Genetic studies on TPH2 have detected several SNPs that are associated with bipolar disorder (Harvey, Shink et al. 2004; Van Den Bogaert, Sleegers et al. 2006; Harvey, Gagne et al. 2007; Lin, Chao et al. 2007; Lopez, Detera-Wadleigh et al. 2007; Cichon, Winge et al. 2008; Roche and McKeon 2009; Campos, Miranda et al. 2011). Furthermore, the SNP C2755A can encode for a S41Y substitution in the regulatory domain of the TPH2 protein, affecting enzyme phosphorylation and resulting in reduced enzyme stability and activity (Carkaci-Salli, Salli et al. 2014). Interestingly, comorbidity with other conditions like major depression, suicidal behaviors and panic disorder was detected with some SNPs in

the TPH2 gene, indicating the complex nature of the behavioral effects that can be controlled by TPH2 expression (Van Den Bogaert, Sleegers et al. 2006; Lin, Chao et al. 2007; Lopez, Detera-Wadleigh et al. 2007; Campos, Miranda et al. 2011).

1.4.1.3 Suicidal behavior

Suicidal behavior can be manifested as part of the behavioral spectrum in affective disorders, particularly in major depression and bipolar disorder. Nevertheless, comorbidity with other psychiatric disorders is common (Hawton and van Heeringen). SNP and haplotype analyses of the TPH2 gene in suicide victims and patients with suicide attempts have also revealed an association of TPH2 with suicidal behavior (Zill, Buttner et al. 2004; Ke, Qi et al. 2006; Jollant, Buresi et al. 2007; Lopez de Lara, Brezo et al. 2007; Lopez, Detera-Wadleigh et al. 2007; Yoon and Kim 2009). Furthermore, increase in TPH2 mRNA transcript levels was observed in dorsal raphe of suicide victims (Bach-Mizrachi, Underwood et al. 2006). Taken together, these findings suggest that changes in TPH2 levels within the brain may influence the decisions and behaviors leading to suicide.

1.4.1.4 Attention-deficit hyperactivity disorder

Attention-deficit hyperactivity disorder (ADHD) is a common neuropsychiatric condition diagnosed in children and adolescents, and is typically characterized by short attention spans, hyperactivity or

restlessness, and impulsive and disruptive behaviors (Polanczyk, de Lima et al. 2007). Since the identification of TPH2, several studies have associated polymorphisms of this neuronal specific TPH to ADHD (Sheehan, Lowe et al. 2005; Walitza, Renner et al. 2005; Brookes, Xu et al. 2006; Lasky-Su, Neale et al. 2008; Manor, Laiba et al. 2008; Park, Park et al. 2013), indicating that TPH2 can influence the changes in cognitive processes and motor activity underlying the behaviors observed in ADHD patients. In addition, a functional TPH2 SNP C907T has also been identified to be associated with ADHD. This SNP encodes a missense R303W mutation in TPH2, resulting in decreased enzyme solubility and activity *in vitro*. (McKinney, Johansson et al. 2008)

1.4.2 Schizophrenia

Schizophrenia is a psychiatric disorder where patients can both exhibit negative symptoms such as abnormal social behavior, inactivity and lack of motivation, as well as positive symptoms like delusions, confusion in thinking and hallucinations. As dysregulation of serotonin signaling has been related to schizophrenia, genetic studies have been performed to determine whether TPH2 has a direct role in the pathophysiology of schizophrenia. To date, genetic association between TPH2 and schizophrenia is still relatively inconclusive. Several studies have shown that SNPs in the TPH2 gene are associated with schizophrenia or its positive symptoms (Zhang, Li et al.

2011; Schuhmacher, Becker et al. 2012; Xu, Ding et al. 2014). On the other hand, a number of reports did not find associations between TPH2 SNPs and schizophrenia (Shiroiwa, Hishimoto et al. 2010; Tee, Chow et al. 2010; Kim and Yoon 2011; Watanabe, Egawa et al. 2012). Nevertheless, these conflicting findings may be due to the heterogeneous nature of schizophrenia and the population analyzed. Moreover, differences in methodologies and sample sizes may also contribute to the discrepancy.

1.4.3 Panic disorder

Panic disorder is another complex neuropsychiatric condition that is manifested through anxiety attacks. In addition to affective disorders and schizophrenia, associations of TPH2 SNPs with panic disorders have been detected (Maron, Toru et al. 2008; Kim, Lee et al. 2009). While contradictory findings have also been reported (Mossner, Freitag et al. 2006), the heterogeneous nature of neuropsychiatric disorders and the role of TPH2 in these conditions are further reiterated.

1.4.4 Autism

Autism is a neurodevelopmental disorder that, like ADHD, is a condition more commonly diagnosed in children. Autistic patients typically exhibit impaired social behaviors as well as repetitive behavior, which can be influenced by 5-HT. As TPH2 controls brain 5-

HT synthesis, genetic studies on TPH2 have also been carried out to determine whether TPH2 is associated with autism. Several association studies have suggested that TPH2 polymorphisms are related to autism (Coon, Dunn et al. 2005; Yang, Yoo et al. 2012; Egawa, Watanabe et al. 2013; Singh, Chandra et al. 2013). Nevertheless, conflicting results have also been reported (Sacco, Papaleo et al. 2007). This could be due to differences in methodology and the populations sampled, compounded by the complex nature of the disorder.

1.5 OBJECTIVES OF STUDY AND HYPOTHESES

Current treatment of neuropsychiatric disorders that involve the brain serotonergic system includes drugs like selective serotonin reuptake transporters (SSRIs) and monoamine oxidase inhibitors (MAOIs) (Veenstra-VanderWeele, Anderson et al. 2000). This effectively increases 5-HT signaling by preventing 5-HT reuptake or breakdown, respectively. 5-HT receptor agonists and antagonists, such as atypical antipsychotics, can also alter downstream signaling pathways by modulating the effects of 5-HT. However, these treatments often result in undesirable side effects in patients, such as nausea, insomnia and even sexual dysfunction, possibly due to their action on the peripheral system (Stahl 1998; Yamada and Yasuhara 2004). With the discovery of TPH2 and its implications in these psychiatric disorders, this neuronal-specific isoform of TPH may play a

more specific role in the regulation of 5-HT levels in the brain. As TPH2 presents an attractive target for the development of improved therapeutic interventions with fewer side effects, it is important to elucidate regulatory mechanisms of this rate-limiting enzyme. This in turn would allow the development of more effective treatments and diagnosis of psychiatric disorders in the near future.

As such, the focus of this work is to study the regulatory pathways that are involved in regulating TPH2 protein expression and activity. Specifically, we aim to identify novel regulators of TPH2 that may affect its stability, as well as elucidate mechanisms that can give rise to the instability of TPH2 *in vitro*. Since protein-protein interaction analyses of TPH2 using the whole human proteome has yet to be performed, we postulated that additional TPH2 interacting partners may exist, in addition to 14-3-3 proteins. We also speculated that differences in amino acid sequences of the TPH1 and TPH2 regulatory domains could result in differential interaction with binding partners. In addition, previous *in vitro* studies have also established the instability and rapid degradation of TPH2, although the degradation pathway was yet to be determined. We postulated that TPH2 could undergo degradation through the ubiquitin proteasome pathway, since TPH1 has been reported to undergo ubiquitination (Kojima, Oguro et al. 2000). Moreover, we hypothesized that both TPH isoforms could be ubiquitinated to different extents, possibly as a result of their differential

regulatory domains, thus giving rise to their differences in stability *in vitro*. These hypotheses will be further investigated in chapters 2 and 3. We believe the findings in this present study will provide new insights into the mechanisms underlying TPH2 stability, which in turn can contribute to the development of novel therapeutic approaches that can improve the stability of TPH2, and ultimately increase brain 5-HT synthesis.

2. THE REGULATION OF TRYPTOPHAN HYDROXYLASE-2 BY HEAT SHOCK PROTEIN HSP70

2.1 INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT) is a major monoamine neurotransmitter that is synthesized from L-tryptophan in a two-step enzyme reaction. The first and rate-limiting step of this reaction is catalyzed by tryptophan hydroxylases (TPH), which are expressed in serotonergic neurons in the central nervous system as well as in peripheral tissues such as intestinal enterochromaffin cells, thrombocytes and mast cells of connective tissues (Veenstra-VanderWeele, Anderson et al. 2000; Walther and Bader 2003; Walther, Peter et al. 2003). Two isoforms of TPH have been identified, and are encoded by two independent genes (Walther and Bader 2003). TPH1 is predominantly expressed and controls 5-HT synthesis in the periphery, while TPH2 is neuronal-specific and regulates 5-HT synthesis in the brain (Zhang, Beaulieu et al. 2004). Previous efforts made to characterize both isoforms have revealed that while TPH1 and TPH2 have highly similar catalytic and tetramerization domains, their N-terminal regulatory domains are divergent. In particular, the first 41 amino acids of TPH2 are unique to the isoform (Walther and Bader 2003). It has been suggested that the dissimilarity in their regulatory domains can give rise to differences in enzymatic properties and regulation of protein stability. Indeed, TPH2 has lower protein

expression levels and undergoes degradation at a faster rate compared to TPH1 (Murphy, Zhang et al. 2008). In addition, the instability and lower solubility of TPH2, when purified *in vitro*, were attributed to the first 150 amino acids that form the TPH2 N-terminal regulatory domain (McKinney, Knappskog et al. 2004; McKinney, Knappskog et al. 2005; Carkaci-Salli, Flanagan et al. 2006).

Similar to other amino acid hydroxylases, the regulatory domains of TPH1 and TPH2 act to control enzyme expression, activity and stability, and thus are involved in regulating 5-HT synthesis (Fitzpatrick 1999). Earlier studies on the regulatory mechanisms of TPH2 have demonstrated that post-translational phosphorylation of specific amino acid residues in the regulatory domain of TPH2 can stabilize its expression and activity (McKinney, Knappskog et al. 2005; Murphy, Zhang et al. 2008). The phosphorylation of TPH2 was also shown to allow interaction with 14-3-3 proteins, which in turn increases enzyme stability (Winge, McKinney et al. 2008). While the role of phosphorylation and protein-protein interactions in the regulation of TPH2 expression and stability are beginning to be understood, additional modifications and protein regulators may exist, but have yet to be elucidated.

Heat shock protein 70 (HSP70) is a family of highly conserved 70 kDa molecular chaperones, which includes the stress inducible

HSP70 (HSP70-1, HSPA1A) and constitutively expressed heat shock cognate 70 (HSC70, HSPA8). When induced as part of the stress response, HSP70 proteins are able to prevent the aggregation and degradation of proteins, in turn improving protein stability during stress (Mayer and Bukau 2005). In addition, HSP70 proteins can aid the folding and subcellular transport of newly synthesized proteins, and also play a role in protein degradation under non-stress conditions (Chiang, Terlecky et al. 1989; Bercovich, Stancovski et al. 1997; Frydman 2001; Pratt and Toft 2003). Consistent with its role in protein homeostasis, HSP70 proteins have been implicated in various diseases, including neurodegeneration, immunomodulation and cancer (Turturici, Sconzo et al. 2011).

In this study, we found a novel TPH-interacting protein, interacting with both TPH1 and TPH2 *in vitro*. Using mass spectrometry, we have identified this protein to be HSP70 (HSP70-1, HSPA1A). A stronger interaction was observed between HSP70 and TPH1, while interaction was weaker between HSP70 and TPH2. Our results also suggested that the main HSP70 interaction domains on both TPH isoforms could be located within the N-terminal regulatory domains. Pharmacological inhibition demonstrated that TPH2 was more sensitive to changes in levels of HSP70, as compared to TPH1. Furthermore, RNA interference indicated that HSP70 functions as a positive regulator of TPH2 enzyme expression and activity, possibly by

stabilizing protein folding. Taken together, our results indicated that the instability of TPH2 was in part due to its poor interaction with HSP70.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Cell culture and reagents

PC12 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum (Gibco, Life Technologies) and 5% bovine calf serum (HyClone, Thermo Scientific). HEK293 cells were maintained in Eagle's minimal essential medium with Earle's salt, supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific) and 0.1% gentamicin solution (Life Technologies). N-Formyl-3,4-methylenedioxy-benzylidine-γ-butyrolactam (KNK437, Calbiochem) was prepared as a 50 mM stock solution in dimethyl sulfoxide. Dimethyl sulfoxide was used as a control in treatment assays. Doxycycline (Clontech, Takara) was prepared in water as a 1 mg/ml stock solution.

2.2.2 Molecular reagents and transfection

HEK293 cells were transiently transfected using calcium phosphate method. Transient transfection in PC12 cells was performed using Lipofectamine 2000 transfection reagent (Life Technologies) according to manufacturer's instructions; or by electroporation, using a Gene Pulser II (Bio-Rad), at 300 V and 1000 µF. All overexpression

constructs were generated with a triple hemagglutinin (HA) tag at the N terminus and inserted into pcDNA3.1 vector (Invitrogen), as previously described (Murphy, Zhang et al. 2008). The doxycycline inducible HA-tagged TPH2 was sub-cloned into pTRE-Tight vector (Clontech) at EcoRV and XbaI sites, and transfected into PC12 cells stably expressing pTet-On vector (Clontech), which contained the reverse tetracycline controlled transactivator (rtTA) gene. shRNA inserts were designed using BLOCK-iT™ RNAi Designer (Invitrogen). Full-length shRNA inserts were generated by short oligomer annealing and T4 polynucleotide kinase (New England BioLabs) treatment, and then cloned into pBluescript SK+ (Stratagene) containing a H1 promoter, at BglII and HindIII sites. All generated constructs were confirmed by sequencing.

2.2.3 Immunoprecipitation and SDS-PAGE

HEK293 cells were collected 48 h post-transfection, and then resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100) in the presence of 5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1x Complete EDTA-free protease inhibitor (Roche), using a pellet homogenizer (Kontes, Fisher Scientific). Lysates were centrifuged at 13200 rpm for 10 min at 4°C, and resulting supernatants were incubated with anti-HA agarose (Sigma-Aldrich) or sepharose A/G beads (Pierce, Thermo Scientific) conjugated to anti-HSP70 antibodies (Stressgen, Enzo Life Sciences)

overnight, on a rotator at 4°C. Beads were washed thoroughly with lysis buffer, and bound proteins were eluted using 1x Laemmli buffer with 10% β-mercaptoethanol. For mass spectrometry, elution of bound proteins was performed using 1 M glycine to reduce the presence of IgG heavy and light chains that may hinder analyses. All eluted proteins were heated at 55°C for 20 min before separation by SDS-PAGE. Immunoprecipitated samples for mass spectrometry analyses were resolved on 10% Ready Gel Tris-HCl precast gels (Bio-Rad) and stained with InstantBlue (Expedeon) according to the manufacturer's instructions. Eluted samples for Western blotting were resolved and immunoblotted as described below.

2.2.4 Western blotting

At 48-72 h post-transfection, or the indicated time points after pharmacological treatment, cells were collected and resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) with 5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1x Complete EDTA-free protease inhibitor (Roche) for lysis. Soluble proteins were separated by centrifugation at 13200 rpm for 10 min at 4°C, and quantified using DC protein assay kit (Bio-Rad). Protein samples were then diluted in 2x Laemmli sample buffer with 10% β-mercaptoethanol and incubated at 55°C for 15 min before electrophoresis. Protein samples were loaded in equal amounts, unless otherwise stated, and

resolved on 10% SDS-PAGE before being transferred onto nitrocellulose membranes (Bio-Rad). Antibodies used for detection of proteins of interest were as follows: anti-HA (Covance Inc.), anti-actin (Millipore), anti-HSP70 (SPA-810, Stressgen, Enzo Life Sciences), and anti-HA Peroxidase (Roche). All primary antibodies were incubated overnight at 4°C. Immunoblots probed with non-peroxidase conjugated antibodies were incubated with horseradish peroxidase conjugated goat anti-mouse secondary antibodies (Pierce, Thermo Scientific). All blots were then developed using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Thermo Scientific), and detected with Fujifilm Super RX-N film or visualized using ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare). For quantification of protein levels, band intensities from at least three independent experiments were quantified using ImageJ software (Version 1.43u, National Institutes of Health).

2.2.5 Measurement of 5-HT levels

PC12 cells stably expressing the rtTA transactivator protein from the pTet-On vector (Clontech) were treated with doxycycline for 18 h, and then counted using a hemocytometer. The cells were washed using phosphate-buffered saline, and resuspended in 0.5 N perchloric acid using a pellet homogenizer (Kontes, Fisher Scientific). The homogenates were separated by centrifugation at 13200 rpm for 5 min at 4°C, and supernatants were filtered using 0.2 µm centrifugal filter

units (Millipore). Filtrates were then analyzed by high pressure liquid chromatography (HPLC) with electrochemical detection. Samples were separated on an ESA microdialysis MD-150 analytical column (3 mm x 15 cm; Thermo Scientific Dionex), with a mobile phase of 90 mM sodium phosphate monobasic dihydrate, 50 mM citric acid, 2.1 mM 1-octanesulfonate monohydrate, 0.1 mM EDTA, and 12.5% acetonitrile at pH 3.0. Electrode potential was applied at +200 mV. 5-HT levels were recorded and quantified within a linear range based on a series of 5-HT standards of known concentrations, then normalized to the cell number within each analyzed sample.

2.2.6 Mass spectrometry

One-step peptide extraction and MALDI-TOF/TOF MS analyses was performed by The Proteomic Core Facility of the Biological Research Center at Nanyang Technological University, as described previously (Meng, Zhang et al. 2008). Briefly, bands of interest were excised and subjected to in-gel digestion with trypsin. Trypsin digestion was then inactivated, and conditioned C18 Empore Disks (3M) were incubated in the digested solution before bound peptides were eluted. Subsequently, MS analyses were carried out using a MALDI-TOF/TOF mass spectrometer (Applied Biosystems). MS and MS/MS data were acquired in combination mode, and the combined MS and MS/MS spectra was processed using GPS Explorer 3.6 (Applied Biosystems) and searched against the Swiss-Prot database using MASCOT 2.0

server. The searches were carried out at a precursor ion tolerance of < 0.15 Da and MS/MS fragment ion error tolerance of < 0.3 Da. Oxidized methionine was included as a variable modification, while carbamidomethyl cysteine was selected as a fixed modification. Proteins with significance scores of $p < 0.05$ were considered as true positives.

2.2.7 Statistical analyses and bioinformatics

Protein sequence alignment was performed using ClustalW2 (EMBL-EBI). Student's two-tailed *t* test was used to compare the differences in HSP70 interaction between TPH1 and TPH1 Δ10 truncated mutant, as well as differences in expression between TPH1 and TPH2 after pharmacological treatment or RNA interference. All other differences in HSP70 interaction were compared using analysis of variance with Tukey's post hoc analysis. Data was considered to be statistically significant when $p < 0.05$. Statistical analyses were performed and all graphs were plotted using GraphPad Prism (Version 5.0d, GraphPad Software).

2.3 RESULTS

2.3.1 HSP70 interacts with both isoforms of TPH

In order to identify interacting partners of TPH, HA-tagged TPH1 and TPH2 were transiently expressed in HEK293 cells.

Immunocomplexes were pulled down using anti-HA agarose, and immunoprecipitated fractions were then resolved on SDS-PAGE. We have observed that, in addition to the bait HA-tagged TPH isoforms, higher molecular weight proteins were also detected in the immunoprecipitated fractions (Figure 2.1A). These proteins were of similar sizes, and co-immunoprecipitated together with HA-TPH1 and HA-TPH2, but not with HA-YFP, indicating that the interaction was specific to both TPH isoforms. MALDI-TOF/TOF MS was used to determine the identity of these higher molecular weight proteins. From two independent experiments, we identified them to be members of the 70 kDa heat shock protein family. Proteins with the highest scores include HSP70, HSP70B', HSC70 and HSPA1L (Figure 2.1B, Table 2.1). As HSP70 is a common interacting protein of both TPH1 and TPH2, we further investigated its interaction and role with respect to the regulation of TPH expression and stability. Immunoprecipitation using anti-HA antibodies and Western blot confirmed that exogenous HA-TPH1 and HA-TPH2 could interact with endogenous HSP70 (Figure 2.1, C and D). To determine whether HSP70 interaction could also occur in the absence of transient overexpression, we generated HEK293 cells lines that stably express HA-TPH1 and HA-TPH2. Endogenous HSP70 was observed to co-immunoprecipitate with HA-TPH1 and HA-TPH2 in these stable HEK293 cells, when pulled down using anti-HSP70 antibodies and detected by Western blot (Figure 2.1, E and F). As little or no protein-protein interactions were observed

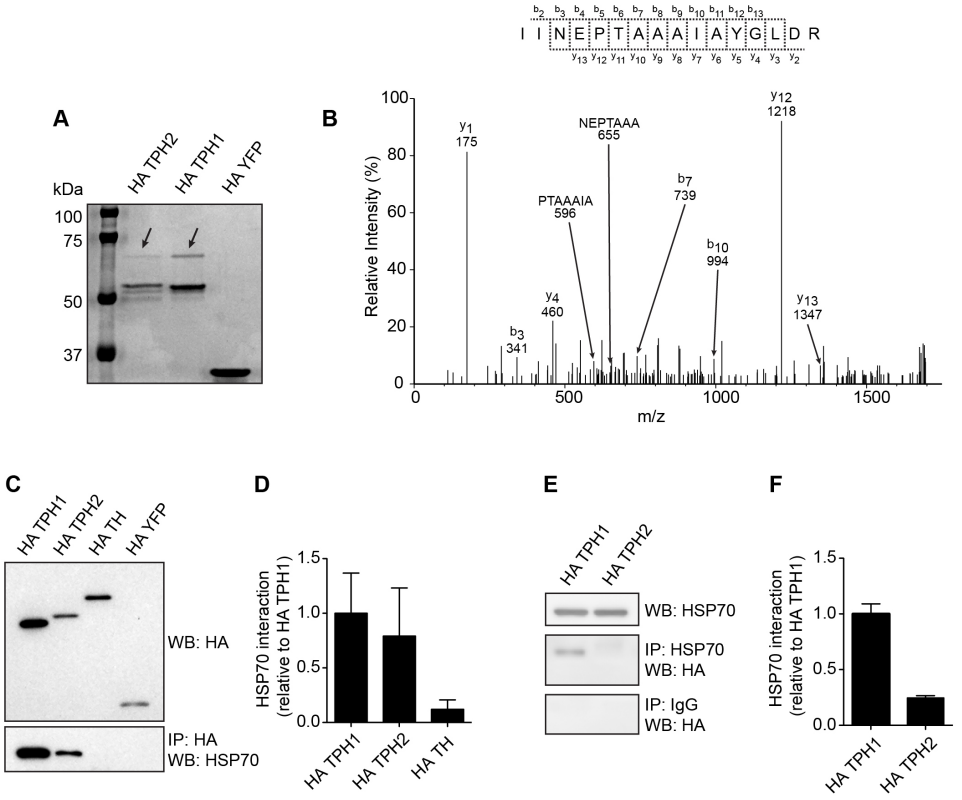

FIGURE 2.1. HSP70 is a novel interacting protein of both TPH isoforms. A, HA-TPH1 and HA-TPH2 were transiently overexpressed in HEK293 cells and immunoprecipitated (IP) to identify interacting proteins. Bands of interest (arrows) were detected using Coomassie Blue and excised from SDS-PAGE, then analyzed by MALDI-TOF. B, A representative MS/MS spectrum of a tryptic peptide fragment from HSP70. C, Interactions between endogenous HSP70 with TPH1 and TPH2 were confirmed using immunoblotting, using HA-TH and HA-YFP as negative controls. 20 µl of HA-TPH1 and HA-TPH2 lysate input fractions, and 2 µl of HA-TH and HA-YFP lysate input fractions were loaded on SDS-PAGE for Western blot. D, Densitometry analysis was performed on immunoblots similar to that in C. Band intensities of HSP70 were normalized to that of the HA-tagged substrates in the IP fractions, and presented as a ratio to HSP70 interaction with HA-TPH1. HA-YFP was not represented in the graph as its interaction with HSP70 was not observed. E, Reciprocal IP using HSP70 antibodies revealed interactions between HSP70 with HA-TPH1 and HA-TPH2 in HEK293 stable cells. F, Quantification of the extents of HSP70 interaction with HA-TPH1 or HA-TPH2 in HEK293 stable cells. Band intensities of HA-tagged substrates were normalized to that of HSP70 in the IP fractions, and presented as a ratio to HSP70 interaction with HA-TPH1; $p = 0.0535$ when HSP70 interaction with HA-TPH2 was compared to that of HA-TPH1. All graphical data are means ± S.E. (n=3).

TABLE 2.1. Higher molecular weight interacting partners of TPH1 and TPH2

Protein Name	Protein Size (MW)	Accession number	Protein Score	Protein Score C.I. %
TPH1 interacting proteins				
Heat shock 70 kDa protein 1 (HSPA1A, HSP70)	70280.1	P08107	264	100.000
Heat shock 70 kDa protein 6 (HSPA6, HSP70B')	71440.4	P17066	123	100.000
Heat shock cognate 71 kDa protein (HSPA8, HSC70)	71082.3	P11142	98	100.000
Heat shock 70 kDa protein 1L (HSPA1L)	70730.5	P34931	60	98.359
TPH2 interacting proteins				
Heat shock 70 kDa protein 1 (HSPA1A, HSP70)	70280.1	P08107	118	100.000

Proteins within excised bands (arrows, from Figure 2.1A) were identified by MALDI-TOF/TOF MS. Alternative names of the proteins were provided within parentheses. Accession numbers were given in UniProtKB/Swiss-Prot format. Only the hits with protein scores above 55 were considered significant ($p < 0.05$) and represented in the table. Confidence levels of the protein scores were reflected as confidence intervals (C.I. %). High C.I.% values denote the correct identification of proteins.

when HA-tagged tyrosine hydroxylase (HA-TH) or HA-YFP were expressed (Figure 2.1, C and D), these results indicated that interactions between HSP70 with TPH1 and TPH2 were specific.

2.3.2 HSP70 interacts with TPH1 primarily through the N-terminal regulatory domain

As TPH1 was found to be a stronger interacting partner with HSP70, we sought to determine the HSP70 interaction domain of TPH1. Comparison of the amino acid sequences of TPH1 and TPH2 revealed that the N-terminal regulatory domains of the two isoforms were not conserved (Walther and Bader 2003) (Figure 2.2A). Furthermore, the differences in the regulatory domains of TPH1 and TPH2 have been shown to give rise to differences in enzyme stability and expression (Murphy, Zhang et al. 2008). As such, we hypothesized that differences in the regulatory domain sequences could result in different extents of interaction with HSP70.

In order to investigate this hypothesis, we have to first determine the interaction site of HSP70. As such, we generated a series of HA-tagged N-terminal deletion mutants of TPH1 (Figure 2.2B). Immunoprecipitation and Western blot demonstrated that when the first ten amino acids in the N-terminal regulatory domain of TPH1 were removed, enzyme expression and interaction with endogenous HSP70 decreased significantly (Figure 2.2, C and D). Subsequent progressive

N-terminal truncations of TPH1 resulted in a more gradual decrease in enzyme expression and HSP70 interaction (data not shown). As such, our results indicated that the first ten amino acids of TPH1 could play a role in HSP70 interaction, and in turn affect enzyme expression and stability.

To further investigate the role of the first ten amino acids of TPH1 (hereby termed as T1) in HSP70 interaction and enzyme stability, chimeric mutants were made (Figure 2.3A). As T1 is not conserved between the two isoforms of TPH, a HA-tagged substitution mutant, HA-TPH2-T1S, was generated. In this mutant construct, amino acids 2-10 of TPH1 replaced their corresponding amino acid sequence, amino acids 43-51, on TPH2 (Figure 2.2A and 2.3A). In addition, we also noted that the potential HSP70 interaction site in TPH2 was not found at the N-terminus like that of TPH1. As such, the first 41 amino acids unique to TPH2 might interfere with the interaction between HSP70 and amino acids 43-51 of TPH2. To address this possibility, we generated a HA-tagged insertion mutant, HA-TPH2-T1E, where amino acids 2-10 of TPH1 were added to the N-terminus of TPH2 (Figure 2.3A). Immunoprecipitation and Western blot were performed using these chimeric mutants to determine expression levels and HSP70 interaction. We observed an increase in protein expression in both chimeric mutants as compared to wild type HA-TPH2, with HA-TPH2-

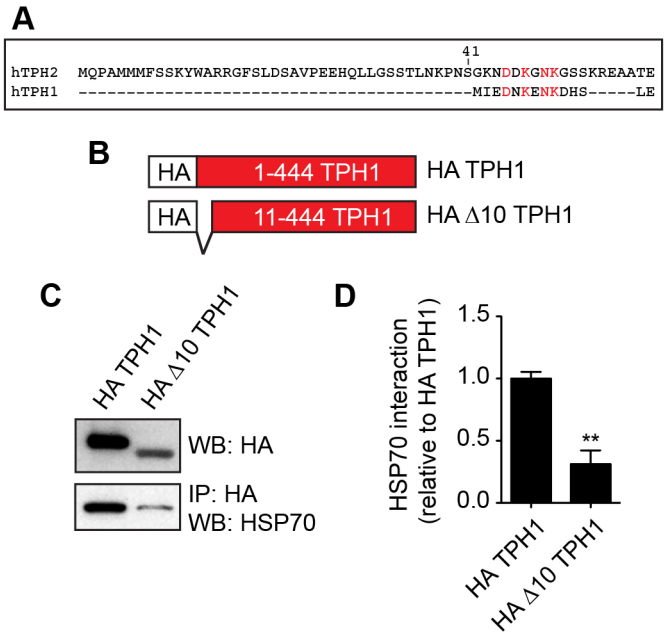

FIGURE 2.2. Mapping the interaction domain of HSP70 in TPH1. A, The N-terminus of TPH1 and TPH2 are divergent, although amino acids 41-51 of TPH2 show some similarity with amino acids 2-10 of TPH1. B, TPH1 N-terminal deletion constructs were made in order to determine the region of interaction with HSP70. C, IP of HEK293 cell lysate overexpressing the TPH1 Δ10 truncated mutant (HA-Δ10-TPH1) indicated that interaction of TPH1 with endogenous HSP70 decreased significantly when the first 10 amino acids of TPH1 were removed. D, Quantification of the extents of HSP70 interaction with HA-TPH1 or HA-Δ10-TPH1. Band intensities of HSP70 were normalized to that of the HA-tagged substrates in the IP fractions, and presented as a ratio to HSP70 interaction with HA-TPH1; **, $p < 0.05$ compared to HSP70 interaction with HA-TPH1. Data are means ± S.E. (n=3).

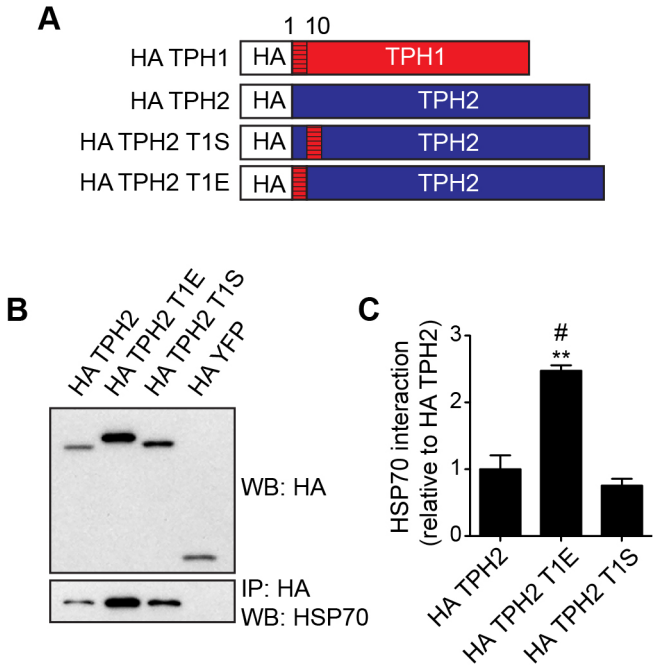

FIGURE 2.3. HSP70 interacts with first 10 amino acids in N-terminus of TPH1. A, Insertion and substitution mutants (HA-TPH2-T1E and HA-TPH2-T1S) were constructed to further investigate the role of the first 10 amino acids in the N-terminal of TPH1 (T1) in HSP70 interaction. B, When transiently overexpressed in HEK293 cells, both T1 mutant constructs exhibited increased expression as compared to HA-TPH2. HA-TPH2-T1E was expressed more efficiently, and also displayed increased HSP70 interaction, as compared to HA-TPH2-T1S. C, Quantification of the extents of HSP70 interaction with HA-TPH2, HA-TPH2-T1E and HA-TPH2-T1S. Band intensities of HSP70 were normalized to that of the HA-tagged substrates in the IP fractions, and presented as a ratio to HSP70 interaction with HA-TPH2. **, $p < 0.05$ compared to HSP70 interaction with HA-TPH2; #, $p < 0.05$ compared to HSP70 interaction with HA-TPH2-T1S. Data are means ± S.E. (n=3).

T1E exhibiting slightly higher expression as compared to HA-TPH2-T1S (Figure 2.3B). Furthermore, quantification of HSP70 interaction revealed that HA-TPH2-T1E was able to interact with HSP70 more strongly as compared to HA-TPH2 or HA-TPH2-T1S (Figure 2.3C). As such, these observations further supported the notion that T1 could play a role in HSP70 interaction. Interaction between TPH2 and HSP70 may be weaker as T1 is not fully conserved in TPH2. Furthermore, the corresponding amino acids to T1 on TPH2 are found after the N-terminal 41 amino acids unique to TPH2, which could prevent interaction with HSP70 as a result of protein folding.

2.3.3 Pharmacological inhibition of HSP70 decreases both TPH1 and TPH2 expression

In order to study the role of HSP70 in regulating TPH1 and TPH2 *in vitro*, we used the stable HEK293 cells lines expressing HA-TPH1 and HA-TPH2 that we have generated. These stable cell lines were treated with KNK437, which has been demonstrated to inhibit the transcriptional activation of HSP70 (Yokota, Kitahara et al. 2000). It was observed that expression of both TPH1 and TPH2 decreased after KNK437 treatment (Figure 2.4A). However, TPH2 expression decreased at a relatively faster rate as compared to that of TPH1 after the cells were treated with KNK437 over a period of 24 h (Figure 2.4, A and B). To determine whether the above effects of KNK437 can be

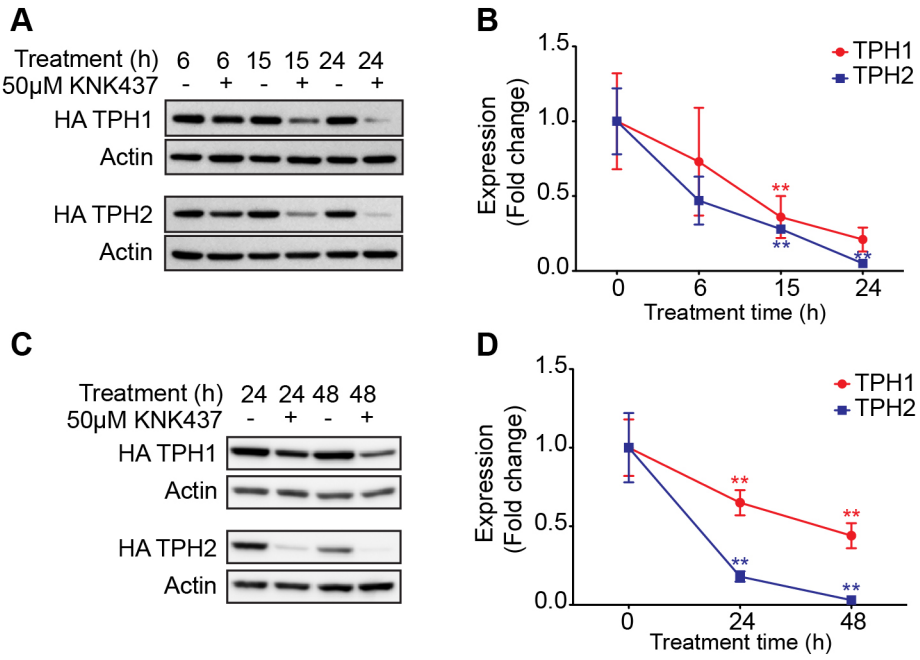

FIGURE 2.4. TPH2 is more sensitive to HSP70 inhibition as compared to TPH1. A, HEK293 cells stably expressing HA-TPH1 and HA-TPH2 were treated with KNK437 for up to 24 h. Western blot showed that TPH1 expression decreased at a slower rate compared to that of TPH2 in response to KNK437 treatment. B, Quantification of changes in TPH1 and TPH2 expression levels in HEK293 stable cells after KNK437 treatment over 24 h. Expression levels were normalized to actin and presented as a ratio to expression levels at the start of treatment; **, $p < 0.05$ compared to expression levels at the start of treatment. Data are means ± S.E. (n=3). C, Similar results were observed when PC12 cells, stably expressing HA-TPH1 and HA-TPH2, were treated with KNK437 over 48 h. D, Quantification of changes in TPH1 and TPH2 expression levels in PC12 stable cells treated with KNK437 over 48 h. Expression levels were normalized to actin and represented as a ratio to expression levels at the start of treatment; **, $p < 0.05$ compared to expression levels at the start of treatment. Data are means ± S.E. (n=3).

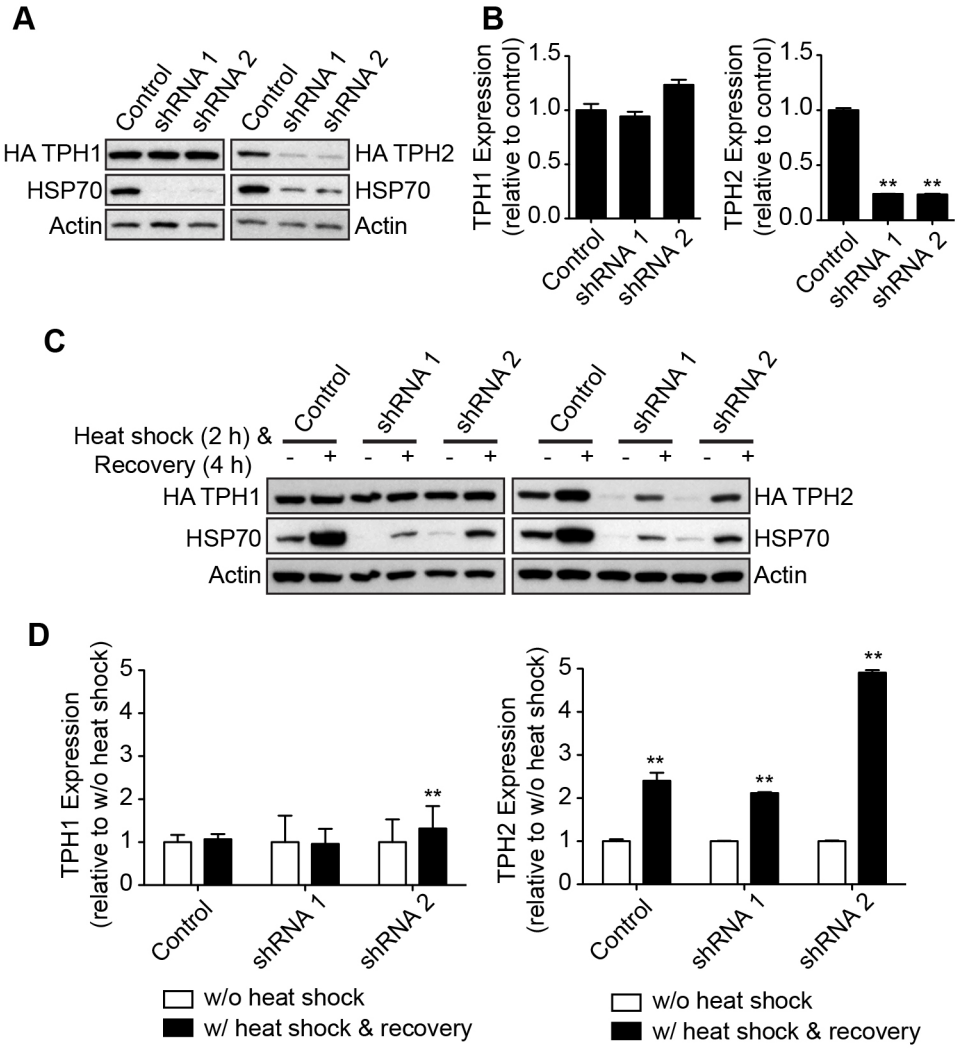

FIGURE 2.5. HSP70 knockdown affected TPH2 expression but not TPH1. A, PC12 cells stably expressing HA-TPH1 and HA-TPH2 were transfected with two individual shRNA constructs against rat HSP70 (shRNA 1 and shRNA 2). Control shRNA was constructed using identical vector backbone with H1 promoter, but without shRNA insert. Western blot revealed a significant decrease in TPH2 expression when HSP70 levels were reduced, although TPH1 levels remained relatively unchanged. B, Quantification of protein expression of TPH1 and TPH2 in PC12 cells after shRNA knockdown of HSP70. TPH1 and TPH2 expression levels were normalized to actin, and represented as a ratio to protein expression of control; **, $p < 0.05$ compared to control. Data are means ± S.E. (n=4). C, The reduction in TPH2 expression was rescued when shRNA transfected cells were subjected to a 2 h heat shock followed by a 4 h recovery period. However, TPH1 expression levels remained unchanged after heat shock and recovery. D, Quantification of protein expression of TPH1 and TPH2 after shRNA knockdown of HSP70, and after heat shock rescue. TPH1 and TPH2 expression levels were normalized to actin, and shown as a ratio to protein expression without heat shock rescue; **, $p < 0.05$ compared to non-heat shocked expression. Data are means ± S.E. (n=4).

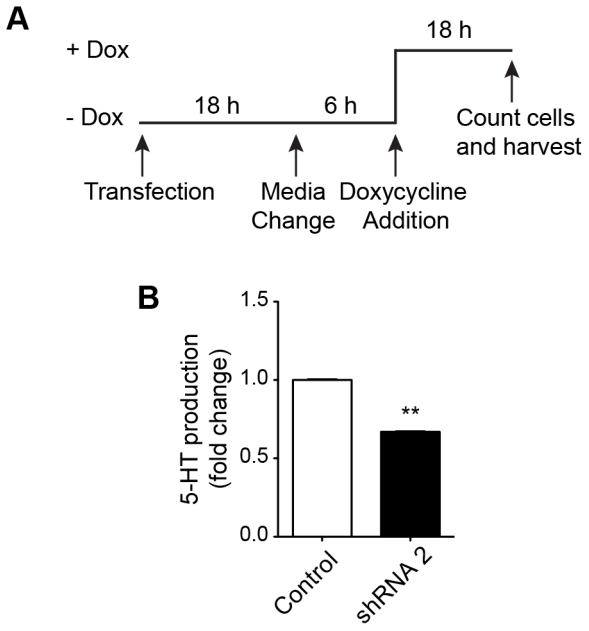

FIGURE 2.6. HSP70 knockdown reduced TPH2 activity. A, PC12 cells transiently overexpressing doxycycline-inducible HA-TPH2, and shRNA against HSP70 (rHSP70 shRNA 2) or control shRNA, were exposed to 1 µg/ml doxycycline (Dox) 24 h after transfection. Doxycycline induction was performed for 18 h, after which the cells were collected for HPLC analyses. B, 5-HT levels induced by doxycycline treatment in PC12 cells were measured by HPLC, then normalized to cell number in each sample. **, $p < 0.05$ compared to PC12 cells transfected with control shRNA. Data are means ± S.E. (n=5).

observed in another cell line, PC12 cells stably expressing HA-TPH1 and HA-TPH2 were also generated. When the PC12 cells were treated with KNK437 over 48 h, we observed a similar decrease in TPH1 and TPH2 expression. However, TPH2 expression decreased significantly at a faster rate as compared to TPH1 (Figure 2.4, C and D). Our results thus indicated that TPH2 is more sensitive than TPH1 to changes in HSP70 levels *in vitro*, which in turn may be attributed to the weaker interaction between TPH2 and HSP70.

2.3.4 HSP70 levels affect TPH2 expression and activity

To further confirm the effects of pharmacological inhibition of HSP70 on TPH expression, and to determine the role of HSP70 in TPH regulation, we used shRNA against HSP70, thus allowing for more specific gene targeting and knockdown. It was observed that in stable PC12 cell lines transfected with two different shRNA constructs against HSP70, TPH2 expression levels were significantly reduced in both cases when HSP70 levels decreased. However, TPH1 expression levels exhibited very little changes when endogenous HSP70 was knocked down (Figure 2.5, A and B).

The effects of HSP70 knockdown on TPH2 expression levels could be rescued with a 2 h heat shock treatment, followed by a 4 h recovery, which transiently increased endogenous HSP70 levels (Figure 2.5, C and D). We also observed that heat shock resulted in an

increase in TPH2 expression levels in mock knockdown cells, further suggesting that an increase in endogenous HSP70 levels would in turn increase the stability and expression of TPH2 *in vitro* (Figure 2.5, C and D). In addition to protein expression, we also examined the effects of HSP70 knockdown on TPH2 activity. To allow efficient control of HA-TPH2 translation after HSP70 knockdown, PC12 cells were first transfected with doxycycline-inducible HA-TPH2 and HSP70 specific or control shRNA. The expression of HA-TPH2 was then induced in the transfected cells by the addition of doxycycline, and the levels of 5-HT produced after 18 h was assessed by HPLC (Figure 2.6A). From Figure 2.6B, 5-HT levels in HSP70 knocked down cells were observed to be about 30% lower as compared to cells with normal levels of HSP70, thus indicating that enzyme activity was also affected when HSP70 levels were reduced. This supported our earlier observations that HSP70 may play a role in stabilizing TPH2, thereby improving enzyme expression and activity.

2.4 DISCUSSION

The present study has revealed a novel *in vitro* interaction between HSP70 and both isoforms of TPH. Although densitometry quantifications did not yield significant results (Figure 2.1, D and F), there was a perceivable trend that TPH1 exhibited a stronger interaction with HSP70, as compared to the interaction between HSP70 and TPH2. It was also demonstrated here that the T1 domain was

primarily responsible for HSP70 interaction in TPH1. However, the potential role of the subsequent residues within the TPH1 regulatory region in HSP70 interaction still requires further investigation. In addition, our results have suggested that amino acids 1-41 of TPH2 may interfere with HSP70 interaction. Nevertheless, whether this effect was due to protein folding as we have proposed, still remains to be established.

It was interesting to note that even though KNK437 treatment reduced TPH1 and TPH2 expression over time, HSP70 knockdown only affected TPH2 expression and activity. KNK437 is a small molecule that inhibits the binding of transcriptional activators, known as heat shock factors, to heat shock sequence elements, the upstream promoter sequences of heat shock genes (Ohnishi, Takahashi et al. 2004). As most heat shock genes are regulated by heat shock factors, it is likely that the transcription of members of other heat shock protein families can also be inhibited during KNK437 treatment (Yokota, Kitahara et al. 2000; Ohnishi, Takahashi et al. 2004; Manwell and Heikkila 2007). Furthermore, other members of the 70 kDa heat shock protein family were also identified as potential interacting partners of TPH1 and TPH2 in this study. Since heat shock proteins are able to form functional complexes with other chaperones and co-chaperones from various heat shock proteins families (Young, Hoogenraad et al. 2003; Meimaridou, Gooljar et al. 2009), it is plausible that HSP70

functions as part of a larger complex of other heat shock proteins that can work collectively to regulate the stability of TPH1 and TPH2. This may also lead to the identification of other potential interacting partners by MALDI-TOF/TOF MS, as we have observed in this study. Nevertheless, the potential partner proteins that we have identified have not been thoroughly investigated, and thus their role in the regulation of TPH1 and TPH2 stability could not be ruled out. Our observations from treating cells with KNK437 also supported this notion that additional chaperones from other heat shock proteins families may play a regulatory role, particularly in TPH1 expression and stability. These chaperones were not detected under the preparation and staining conditions of the samples in this study, possibly because they were present in low levels, or weak interactions might have resulted in dissociation from the heat shock complex. Further studies are required to identify these additional chaperones and determine the mechanism by which they function together with HSP70 to stabilize both isoforms of TPH.

In comparison to KNK437 treatment, RNA interference is a more specific targeting strategy with less off target effects. While TPH1 expression remained relatively unchanged when HSP70 was knocked down, a reduced TPH2 expression was observed. We postulated that this observation could be due to the stronger interaction between TPH1 and HSP70. Since HSP70 knockdown efficiencies in this study were

limited as a result of the constraint in transfection efficiencies, residual levels of endogenous HSP70 might be already sufficient to maintain TPH1 stability and expression. In contrast, slight changes in HSP70 levels would give rise to corresponding changes in TPH2 stability and activity as a result of weaker interaction between TPH2 and HSP70. Moreover, the identification of several other potential interacting partners of TPH1 by MALDI-TOF/TOF MS also presented the possibility that the role of HSP70 in stabilizing TPH1 could be compensated by other heat shock proteins when HSP70 was knocked down. On the other hand, this compensatory mechanism might be absent in TPH2 stabilization, indicating the specificity of the interaction between TPH2 and HSP70.

Taken together, the findings in this study suggested that while HSP70 acted as a chaperone by stabilizing protein expression and activity of both TPH isoforms, its weaker interaction with TPH2 could give rise to an increased sensitivity in TPH2 expression and activity to changes in HSP70, thus increasing the instability of TPH2 *in vitro*. Unlike 14-3-3 proteins, which interact with and stabilize phosphorylated TPH2 (Winge, McKinney et al. 2008), the binding of HSP70 did not require any post-translational modifications. This suggests that the chaperone, as part of a larger heat shock complex, potentially acts on TPH2 as the protein is being translated. However, the binding is relatively weak due to a shielded and non-conserved interacting

domain on TPH2, which may result in protein misfolding and instability during translation.

The dysregulation of brain 5-HT homeostasis and neurotransmission is associated with many neuropsychiatric disorders, such as depression, schizophrenia, and bipolar disorder (Lucki 1998). Since the discovery of TPH2 as the brain specific isoform of the rate-limiting enzyme in 5-HT synthesis, polymorphisms of the TPH2 gene have been identified in cohorts of patients with psychiatric disorders (Zhang, Gainetdinov et al. 2005; Chen, Novak et al. 2006; Hong, Sugawara et al. 2007; Waider, Araragi et al. 2011). This suggests that in addition to signaling through serotonergic receptors, changes in TPH2 enzyme activity may also contribute to the symptoms associated with such disorders, thus making TPH2 a potential therapeutic target. It has become increasingly evident that several regulatory mechanisms exist to modulate TPH2 expression and activity, during transcription, post-transcription, translation, and post-translation (Kuhn, Sakowski et al. 2007; Chen, Vallender et al. 2008; Winge, McKinney et al. 2008; Grohmann, Hammer et al. 2010). This fine control of TPH2 activity may be crucial given the role of brain 5-HT in mood, behavior and cognition. Recent studies have also associated environmental stress with the regulation of TPH2 gene expression. Several studies observed an increase in TPH2 expression following stressful stimuli, which is known to elevate HSP70 levels (Zhang, Takahashi et al. 1994; Matz,

Sundaresan et al. 1996; Brown, Henderson et al. 2006; Sidor, Amath et al. 2010). However, conflicting findings have also reported that exposure to stressful events resulted in reduced TPH2 expression (Rahman and Thomas 2009; Own, Iqbal et al. 2013). This could be due to the tissue specific and species dependant nature of TPH2 expression, as well as cross talk between multiple systemic and cellular components, which contribute to the complex signaling network that mediates the stress response (Clark, Pai et al. 2005; Malek, Sage et al. 2007; Heydendael and Jacobson 2009; Barr and Forster 2011). As the current study only investigated the role of HSP70 in TPH2 expression *in vitro*, the functional significance of the interaction between TPH2 and HSP70, and possibly other heat shock proteins, should also be studied *in vivo*. Nevertheless, with low levels of HSP70 and TPH2 present in the brain under non-stress conditions, *in vivo* interaction may be more difficult to detect (D'Souza and Brown 1998; Bodega, Hernández et al. 2002). Similarly, the role of HSP70 and other heat shock proteins in the regulation of TPH1 can also be further explored in peripheral tissues *in vivo*.

Increased intracellular 5-HT levels have been observed after hyperthermic stress and ischemia (Lin 1997). As HSP70 expression is also induced by heat stress and ischemic conditions following traumatic brain injury (Brown, Rush et al. 1989; Dutcher, Underwood et al. 1998; Sharp, Massa et al. 1999), the current study proposes a potential

mechanism that can associate the increase in TPH2 activity at the cellular level and subsequent brain 5-HT production to the upregulation of HSP70 due to stressful stimuli. While increased 5-HT levels in the brain after injury can give rise to both beneficial and detrimental effects that are still not fully understood, there is increasing evidence that 5-HT is able to induce adult neurogenesis (Brezun and Daszuta 1999; Brezun and Daszuta 2000; Malberg, Eisch et al. 2000; Banasr, Hery et al. 2003). This in turn suggests that 5-HT may be involved in cellular and functional repair. Indeed, antidepressants such as fluoxetine, which can increase tissue levels of 5-HT, have been shown to improve cognitive and motor functions, and also have anti-inflammatory effects in post-ischemic rats (Li, Cai et al. 2009; Lim, Kim et al. 2009). Thus, the stabilization of TPH2 by HSP70 as demonstrated in this study may also provide a possible molecular mechanism of functional repair to damaged neurons in the early stages of stress following acquired brain injury. Interestingly, exercise has also been shown to induce the increase in brain levels of HSP70 and 5-HT (Meeusen and De Meirleir 1995; Gomez-Merino, Béquet et al. 2001; Liu, Yang et al. 2010). Although the functional link between HSP70 and 5-HT in exercise is still being speculated, the molecular regulation of TPH2 stability and 5-HT production by HSP70 presented in this study may provide further insights into exercise endurance and fatigue (Heck, Schöler et al. 2011). Despite these emerging indications, HSP70 may prove to be a difficult therapeutic target due to the promiscuous nature of its

interactions with proteins in both the central nervous system and periphery. Moreover, numerous serotonin receptors and transporters mediate serotonergic signaling in the brain, which may give rise to systemic effects that are complex to resolve *in vivo*. Nevertheless, the findings in this study can contribute new insights into the molecular regulation of TPH2 stability, and further highlight the neuroprotective role of HSP70 in the central nervous system.

3. THE ROLE OF UBIQUITINATION IN REGULATING STABILITY OF TRYPTOPHAN HYDROXYLASE-2

3.1 INTRODUCTION

The role of protein-protein interactions in the regulation of tryptophan hydroxylase-2 (TPH2) stability was explored in the previous chapter. Besides protein regulators, post-translational modifications can also provide an additional level of regulation to allow fine control of TPH2 expression and activity in the brain.

Tryptophan hydroxylases (TPH) catalyze the first and rate-limiting step in the biosynthesis of serotonin (5-HT). Previously considered as a single enzyme, it is now understood that two isoforms exist. Tryptophan hydroxylase-1 (TPH1) controls 5-HT levels in peripheral tissues, while TPH2 regulates the production of 5-HT specifically within the central nervous system (Walther and Bader 2003; Walther, Peter et al. 2003; Zhang, Beaulieu et al. 2004). Characterizations of TPH2 have revealed that the brain specific isoform has an elongated N-terminal regulatory domain, with an additional 41 amino acids that are absent in TPH1 (Walther and Bader 2003; Murphy, Zhang et al. 2008). This extended regulatory domain appears to exert a negative regulatory effect on TPH2, decreasing enzyme solubility and stability *in vitro*, in comparison to TPH1 (Carkaci-Salli, Flanagan et al. 2006; Murphy, Zhang et al. 2008). The lower

expression levels of TPH2 observed in mammalian cells is also attributed to a faster rate of degradation, compared to TPH1 (Murphy, Zhang et al. 2008). Although the two TPH isoforms exhibit distinctive characteristics, both TPH1 and TPH2 can be phosphorylated, similar to other aromatic amino acid hydroxylases (Fitzpatrick 1999). Phosphorylation increases TPH1 and TPH2 solubility, expression and stability *in vitro* (McKinney, Knappskog et al. 2005; Murphy, Zhang et al. 2008). Phosphorylated TPH2 has also been demonstrated to interact with 14-3-3 proteins, which in turn act to stabilize the enzyme (Winge, McKinney et al. 2008). Even though the role of phosphorylation in the regulation of both TPH isoforms has been extensively studied, it is currently still unclear whether post-translational modifications can contribute to the differences in stability that were observed between TPH1 and TPH2 *in vitro*. Nevertheless, the faster rate of degradation of TPH2 may point to the presence of other post-translational modifications in TPH2 that can result in a higher turnover rate as compared to TPH1.

Protein degradation in the cell occurs through two major mechanisms, autophagy and ubiquitination. Autophagy involves the lysosomal degradation of large proteins and organelles by proteases and hydrolases upon the fusion of autophagocytic vesicles with lysosomes. In contrast, proteasomal degradation is an active process that mediates the degradation of most cytosolic proteins (Cooper

2000). Proteins broken down through the proteasomal pathway undergo ubiquitination, which is a post-translational modification process. Ubiquitin is bound onto specific lysine residues of a substrate protein through an ubiquitination cascade involving three types of enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. The resulting poly-ubiquitin chains on the substrate proteins are then able to act as a degradation signal, allowing the transportation of these ubiquitinated proteins to the 26S proteasome, where they are unfolded and broken down (Hershko and Ciechanover 1992; Jentsch 1992). The ubiquitin-proteasome system has been demonstrated to play a role in several neurodegenerative and neurological disorders (Yi and Ehlers 2007; Dennissen, Kholod et al. 2012), and recently has also been implicated in neuropsychiatric disorders, such as schizophrenia and bipolar disorders (Bousman, Chana et al. 2010; Rubio, Wood et al. 2013).

While TPH1 has been reported to undergo ubiquitination (Kojima, Oguro et al. 2000), the degradation mechanism of TPH2 is still not well understood. Given that TPH2 is less stable *in vitro* than TPH1, it is plausible that TPH2 is more heavily ubiquitinated, thus giving rise to a more rapid degradation rate. This present study used a cell culture system to examine the notion of TPH2 ubiquitination, as well as employed site-directed mutagenesis to seek to identify potential ubiquitination sites. The results suggested that heavy ubiquitination

and the subsequent proteolysis of TPH2 could contribute to enzyme instability *in vitro*. Furthermore, our findings also indicated that ubiquitination could occur in the absence of lysine residues, pointing to the potential involvement of alternative mechanisms that could facilitate TPH2 ubiquitination.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Cell culture and reagents

PC12 cells and all stable PC12 cell lines were grown in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum (Gibco, Life Technologies) and 5% bovine calf serum (HyClone, Thermo Scientific). A stock solution of 10 mM N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide (MG132, Tocris Bioscience) was made in dimethyl sulfoxide. Dimethyl sulfoxide was used as a control for all MG132 treatments.

3.2.2 Molecular reagents and transfection

Cells were transiently transfected either by electroporation, at 300 V and 1000 µF using a Gene Pulser II (Bio-Rad), or by Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions. In order to allow for efficient detection of overexpressed proteins, all constructs used in this study were cloned into the

pcDNA3.1 vector (Invitrogen), with either a triple hemagglutinin (HA) tag or DYKDDDDK (FLAG) tag at the N-terminus. Human ubiquitin (NM_018955) was amplified from total cDNA of HEK293 cells by PCR. Single lysine to arginine site-directed mutagenesis was performed using the primers presented in Table 2. KR mutagenesis constructs consisting of multiple lysine to arginine substitutions were generated using synthetic gBlocks gene fragments (Integrated DNA Technologies), except HA-K11-116R and HA-K11-183R which were generated using primers in Table 3.2. All clones generated were verified by sequencing.

TABLE 3.1. List of primers used for single lysine to arginine mutagenesis

K11R-F	ATCAGATATCATGCAGCCAGCAATGATGATGTTTTCCAGT**AGA**TAC
K38R-F	AGCTCAACACTAAAT**AGA**CCTAACTCTGGCA
K38R-R	TGCCAGAGTTAGG**TCT**ATTTAGTGTTGAGC
K43R-F	ACCTAACTCTGGC**AGA**AATGACGACAAAG
K43R-R	TGCCTTTGTCGTCATT**TCT**GCCAGAGTTAG
K54R-F	ACAAGGGAAGCAGC**AGA**CGTGAAGC
K54R-R	TCGGTAGCAGCTTCACG**TCT**GCTGC
K63R-F	TGCTACCGAAAGTGGC**AGG**ACAG
K63R-R	ACAACTGCTGT**CCT**GCCACTTTCG
K116R-R	AGCTCATTGAATTCTGT**TCT**CCCACACTC
K183R-F	TGATGCTGACCACCCAGGATTT**AGG**GACAATGTC
K183R-R	ACATTGTC**CCT**AAATCCTGGGTGGTCAGCATC

Codons corresponding to substituted lysine residues are highlighted in bold. F, forward; R, reverse. All primers are presented in a 5' to 3' direction.

3.2.3 Immunoprecipitation

48 h after transfection and 8 h after MG132 treatment, PC12 cells were collected and homogenized in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100) with 5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1x Complete EDTA-free protease inhibitor (Roche), by a pellet homogenizer (Kontes, Fisher Scientific). Cell lysates were separated by centrifugation at 13200 rpm for 10 min at 4°C, before supernatants were incubated with anti-HA agarose (Sigma-Aldrich) overnight at 4°C with rotation. Beads were washed intensively with lysis buffer. Proteins were then eluted by the addition of 1x Laemmli buffer containing 10% β-mercaptoethanol, followed by heating at 55°C for 20 min. Samples were heated at 55°C for 15 min before equal volumes were resolved by electrophoresis for Western blotting, as described below.

3.2.4 Western blotting

PC12 cells were collected and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), with the addition of 5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1x Complete EDTA-free protease inhibitor (Roche). Lysates were then centrifuged at 13200 rpm for 10 min at 4°C to obtain soluble proteins. All cell lysates obtained were quantified using DC protein assay kit (Bio-Rad), and diluted in 2x Laemmli sample buffer with 10% β-mercaptoethanol.

Protein samples were heated at 55°C for 15 min before being resolved on 10% SDS-PAGE. Transfers onto nitrocellulose membranes were performed using the Mini Trans-Blot Module (Bio-Rad). HA-tagged proteins were examined using anti-HA antibody (Covance Inc.), while FLAG-tagged proteins were analyzed using anti-FLAG M2-Peroxidase (Sigma-Aldrich). Anti-actin (Millipore) was used as control. All immunoblots were incubated with primary antibodies at 4°C overnight. Membranes were incubated with horseradish peroxidase conjugated goat anti-mouse secondary antibodies (Pierce, Thermo Scientific), when non-peroxidase conjugated primary antibodies were used. All blots were then detected using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Thermo Scientific), with Fujifilm Super RX-N film or ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare). Band intensities from at least three independent experiments were used for quantification of protein expression levels, using ImageJ software (Version 1.43u, National Institutes of Health).

3.2.5 Bioinformatics and statistical analyses

Prediction of ubiquitination sites was performed using online predictors UbPred ((Radivojac, Vacic et al. 2010), http://www.ubpred.org/), BDM-PUB (http://bdmpub.biocuckoo.org/index.php) and hCKSAAP_UbSite ((Chen, Zhou et al. 2013),

http://protein.cau.edu.cn/cksaap_ubsite/index.php). ClustalW2 (EMBL-EBI) was used to carry out protein sequence alignment. Student's two-tailed *t* test was applied to compare the protein expression of TPH1 and TPH2 with and without MG132 treatment, and the differences in protein expression between TPH2 and the lysine-less TPH2 mutant. Differences in protein expression levels of wild type TPH2 and lysine to arginine mutants were compared using analysis of variance with Tukey's post hoc analysis. Statistical significance was recognized when $p < 0.05$. GraphPad Prism (Version 5.0d, GraphPad Software) was used to perform statistical analyses and plot all graphs.

3.3 RESULTS

3.3.1 TPH2 expression is regulated by the proteasome

As proteasome inhibitors allow the accumulation of ubiquitinated proteins by preventing proteosomal function, the proteasome inhibitor MG132 was used to investigate the role of the ubiquitin-proteasome system in the degradation of TPH2. PC12 cells stably expressing HA-tagged TPH2 were treated with 1 μM MG132 for 8 h. The cells were then harvested, and TPH2 expression levels were analyzed. TPH2 expression level was observed to increase significantly after MG132 treatment (Figure 3.1, A and B). Similarly, when transiently overexpressed in PC12 cells, TPH2 protein levels also increased after MG132 treatment (Figure 3.1, C and D). In addition to the rise in protein expression levels, higher molecular weight bands

were also detected when cells expressing TPH2 were exposed to MG132. These bands appeared to increase in multiples of approximately 8 kDa, which corresponded to the size of ubiquitin (Figure 3.1, A and C). TPH1 expression also increased after MG132 treatment, albeit to a smaller extent compared to TPH2. However, the distinct higher molecular weight bands were not detected in MG132 treated cells transiently expressing TPH1 (Figure 3.1C). Thus, the results suggested that the degradation of TPH2 could occur through a proteasome dependent pathway.

3.3.2 TPH2 is highly ubiquitinated compared to TPH1

Protein targeting by ubiquitination is required for proteasomal degradation. As such, we sought to determine whether TPH2 could be ubiquitinated. Human ubiquitin (Ub) was cloned and fused with a HA or FLAG tag at its N-terminus. PC12 cells were then transiently transfected with HA-tagged TPH2 together with FLAG-tagged Ub, or FLAG-tagged TPH2 with HA-tagged Ub. The transfected PC12 cells were then treated with MG132 for 8 h, and cell lysates were subjected to immunoprecipitation using HA agarose. As demonstrated in Figure 3.2A, FLAG-Ub was co-immunoprecipitated with HA-TPH2 in MG132 treated cells, but not in the DMSO treated controls. A reciprocal immunoprecipitation of HA-Ub also revealed that FLAG-TPH2 was associated with ubiquitin in the presence of MG132, thus indicating that TPH2 was able to undergo ubiquitination in PC12 cells.

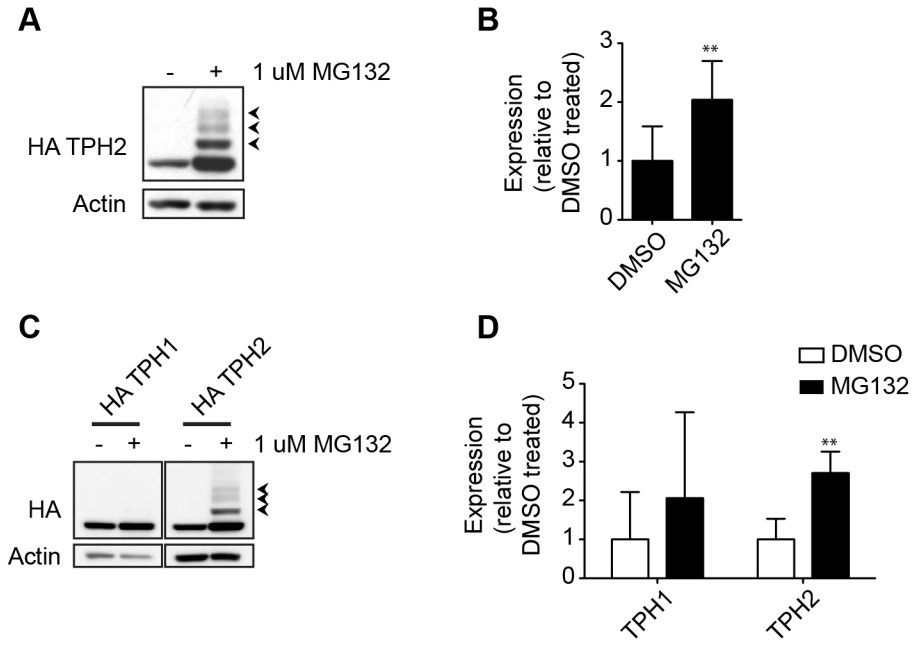

FIGURE 3.1. TPH2 expression is stabilized by proteasomal inhibition. A, PC12 cells stably expressing HA-TPH2 were treated with 1 μM MG132 for 8 h. Western blot revealed an increase in TPH2 protein expression after MG132 treatment, together with distinct bands of higher molecular weight that were increasing in multiples of approximately 8 kDa (arrowheads). B, Quantification of protein expression of TPH2 in PC12 stable cells with or without MG132 treatment. TPH2 expression levels were normalized to actin, and presented as a ratio to protein expression of DMSO treated sample; **, $p < 0.05$ compared to DMSO treated sample. Data are means ± S.E. (n=3). C, In transiently transfected PC12 cells, a similar increase in HA-TPH2 protein expression was observed in MG132 treated cells, in addition to the presence of higher molecular weight bands (arrowheads). HA-TPH1 expression also increased marginally with MG132 treatment, but higher molecular weight bands were not observed. 30 μg of HA-TPH1 expressing cell lysates and 50 μg of HA-TPH2 expressing cell lysates were loaded on SDS-PAGE for Western blot. D, Quantification of protein expression of TPH1 and TPH2, with or without MG132 treatment. TPH1 and TPH2 expression levels were normalized to actin, and shown as a ratio to protein expression of DMSO treated samples; **, $p < 0.05$ compared to DMSO treated sample. Data are means ± S.E. (n=3).

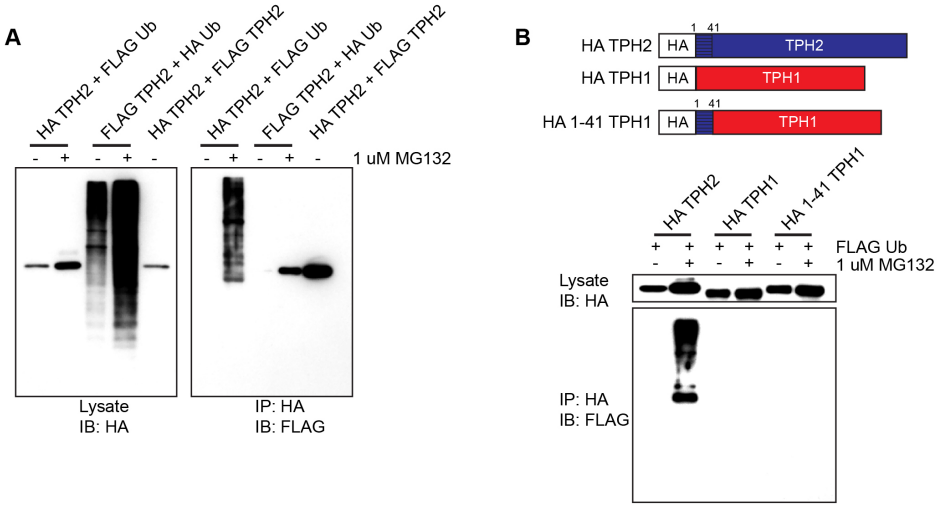

FIGURE 3.2. TPH2 is ubiquitinated more heavily as compared to TPH1. A, PC12 cells were transiently transfected with HA-TPH2 and FLAG-Ub, or FLAG-TPH2 and HA-Ub. Lysates were immunoprecipitated (IP) with HA agarose beads and separated fractions were detected using anti-FLAG on Western blot. FLAG-Ub was observed to associate with HA-TPH2 in MG132 treated cells. Reciprocal IP of HA-Ub also co-immunoprecipitated FLAG-TPH2 in cells treated with MG132. As TPH2 monomers can tetramerize *in vitro*, a transient co-transfection of HA-TPH2 and FLAG-TPH2 served as a positive control. B, FLAG-Ub was similarly co-transfected in PC12 cells with HA-TPH2, HA-TPH1 or chimeric HA-1-41-TPH1, and transfected cells were subjected to MG132 treatment. Subsequent IP and Western blot revealed that TPH2 strongly interacted with FLAG-Ub, as compared to HA-TPH1 or HA-1-41-TPH1.

To further investigate the extent of TPH2 ubiquitination in comparison to TPH1, HA-tagged TPH1 and TPH2 were transiently overexpressed in PC12 cells, together with FLAG-Ub. In addition, the role of the first 41 amino acids unique to TPH2 in ubiquitination was examined, using a HA-tagged chimeric construct that consisted of the first 41 amino acids of TPH2 added to the N-terminal of TPH1 (HA-1-41-TPH1, Figure 3.2B). This chimeric protein was also co-expressed in PC12 cells together with FLAG-Ub. Cells were incubated in the presence or absence of MG132 for 8 h, and lysates were then immunoprecipitated using HA agarose. When the immunoprecipitated fractions were analyzed by Western blot, it was noted that while FLAG-Ub interacted with HA-TPH2 in MG132 treated cells, very low amounts of FLAG-Ub was co-immunoprecipitated with HA-TPH1 and HA-1-41-TPH1, even after MG132 treatment (Figure 3.2B). This suggested that TPH2 was more heavily ubiquitinated compared to TPH1, and also indicated that the bulk of TPH2 ubiquitination did not occur on the first 41 amino acids of its N-terminal regulatory domain.

3.3.3 Regulatory and catalytic domains of TPH2 can be ubiquitinated

As the N-terminal regulatory domains of TPH1 and TPH2 were not conserved, the variation in amino acid sequences may also result in differential ubiquitination and enzyme stability. We identified six lysine residues in the regulatory domain of TPH2 that were not

conserved in TPH1 (Figure 3.3A), which could be potential sites of ubiquitination. In order to examine the roles of these non-conserved lysine residues in the regulation of TPH2 degradation, site-directed mutagenesis was performed to replace these six lysine residues with arginine. HA-tagged mutant constructs with single lysine to arginine substitutions (K11R, K38R, K43R, K54R, K63R and K116R), as well as K11-116R where all six lysine residues were replaced, were then transiently expressed in PC12 cells to determine protein expression and extent of ubiquitination. The expression levels of all substitution mutants were observed to be similar to wild type TPH2 (Figure 3.3, B and C). Furthermore, all mutants also associated with FLAG-Ub in a similar manner to wild type TPH2, when overexpressed together with FLAG-Ub in MG132 treated PC12 cells (Figure 3.3D). This suggested that other lysine residues, rather than the six non-conserved lysine residues identified, could be responsible for the ubiquitination of TPH2.

Taking into consideration that TPH2 has thirty-five lysine residues amongst its entire amino acid sequence, narrowing down the regions of ubiquitination may facilitate the process of identifying ubiquitinated lysine residues. Thus, the ubiquitination of the regulatory and catalytic domains of TPH2 was investigated. HA-tagged constructs of the TPH2 N-terminal regulatory domain (HA-N150), as well as a truncated TPH2 with no regulatory domain (HA-ΔN150-TPH2), were

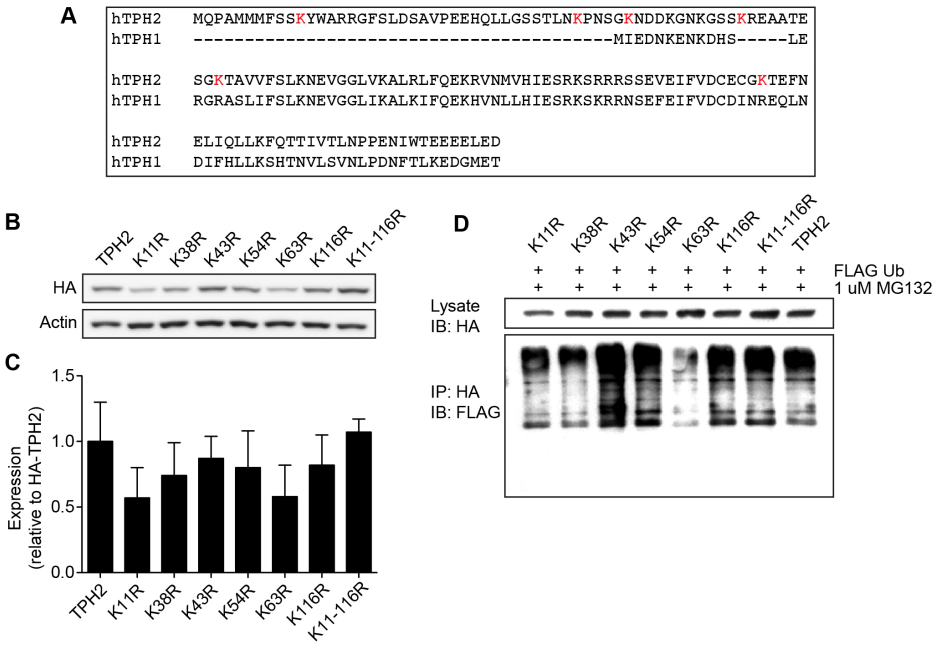

FIGURE 3.3. Mutagenesis of non-conserved lysine residues in the TPH2 regulatory domain did not prevent ubiquitination. A, Protein sequence alignment of both TPH1 and TPH2 N-terminal regulatory domains revealed six lysine residues that are not conserved between the two isoforms (highlighted in red). B, Site-directed mutagenesis was performed at the six sites to generate individual HA-tagged lysine to arginine mutants (K11R, K38R, K43R, K54R, K63R and K116R), as well as a mutant with all six lysines substituted with arginine residues (K11-116R). Mutagenesis constructs were transiently transfected into PC12 cells, and expression levels were assessed by Western blot. C, Quantification of expression levels of all mutagenesis constructs when overexpressed in PC12 cells. Expression levels were normalized to actin, and represented as a ratio to protein expression of wild type TPH2. Data are means ± S.E. (n=3). D, All mutagenesis constructs were co-expressed with FLAG-Ub in PC12 cells, which were then treated with MG132. IP and Western blot showed that FLAG-Ub could still associate with the six individual lysine to arginine mutants, as well as K11-116R.

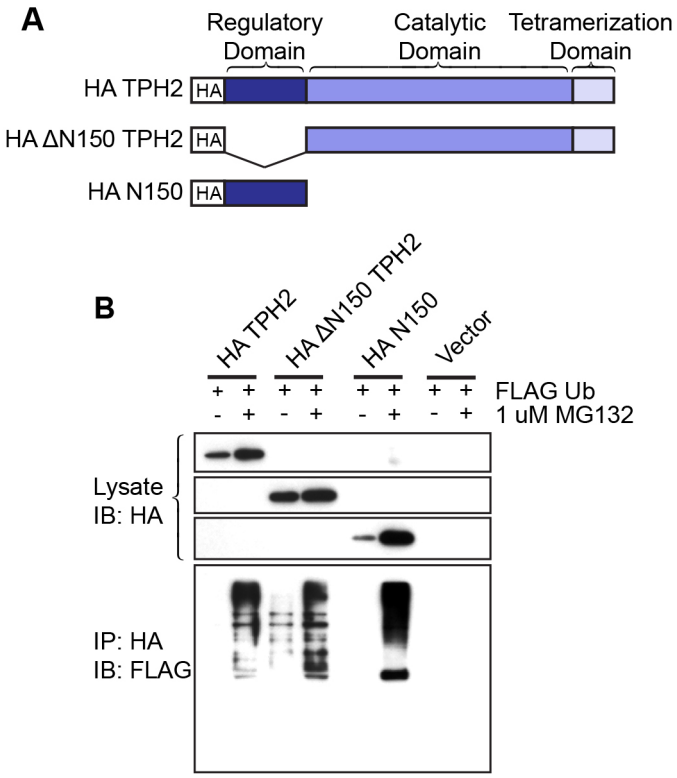

FIGURE 3.4. Determining ubiquitinated domains on TPH2. A, HA-tagged truncation mutants of the TPH2 catalytic and C-terminal tetramerization domains (HA-ΔN150-TPH2), as well as N-terminal regulatory domain (HA-N150) were generated. B, When co-expressed in PC12 cells with FLAG-Ub and exposed to MG132 treatment, both HA-ΔN150-TPH2 and HA-N150 were observed to co-immunoprecipitate with FLAG-Ub, as detected on Western blot.

generated (Figure 3.4A). These constructs were transiently co-expressed with FLAG-Ub in PC12 cells, which were then treated with MG132 before cell lysates were subjected to immunoprecipitation using HA agarose. From Figure 3.4B, expression levels of both HA-N150 and HA-ΔN150-TPH2 was observed to increase after MG132 treatment, similar to TPH2. In addition, FLAG-Ub also associated with both HA-N150 and HA-ΔN150-TPH2 in the presence of MG132, suggesting that multiple ubiquitination sites were present in both the regulatory and catalytic domains of TPH2.

Since multiple ubiquitination sites could be present on TPH2, prediction software was employed to analyze the TPH2 amino acid sequence for potential ubiquitination sites. Two predictors with different algorithms for prediction were used. While the predicted sites varied between the two predictors, lysine residue K183 was found to be a common predicted ubiquitination site, in addition to K54 and K63, which have been previously identified (Table 3.2). As such, we generated an additional HA-tagged mutant construct, where the seven identified lysine residues, including K183, were replaced with arginine (HA-TPH2-K11-183R, Figure 3.5A). When transiently transfected in PC12 cells, the expression of HA-TPH2-K11-183R was relatively unchanged compared to wild type TPH2 and HA-TPH2-K11-116R (Figure 3.5, B and C). Moreover, HA-TPH2-K11-183R associated with FLAG-Ub when both constructs were co-expressed in MG132 treated

PC12 cells (Figure 3.5F). To further understand how these seven lysine-to-arginine mutations affect changes in expression and ubiquitination in the regulatory and catalytic domains, they were introduced into HA-N150 and HA-ΔN150-TPH2 at their respective positions to generate HA-N150-K11-116R and HA-ΔN150-TPH2-K183R (Figure 3.5A). These mutant constructs were transiently expressed in PC12 cells and their expressions were analyzed. A discernible trend was observed, where HA-N150-K11-116R displayed higher expression levels compared to HA-N150, while HA-ΔN150-TPH2-K183R exhibited lower expression compared to HA-ΔN150-TPH2 (Figure 3.5, D and E). Nevertheless, both HA-N150-K11-116R and HA-ΔN150-TPH2-K183R were able to interact with FLAG-Ub when constructs were overexpressed in MG132 treated PC12 cells (Figure 3.5F). Taken together, the data presented in this section indicated that mutation of K11, K38, K43, K54, K63, K116 and K183 were not sufficient to abolish ubiquitination and proteasomal degradation of TPH2. This in turn suggested that ubiquitination could occur on numerous sites located in both the regulatory and catalytic domains of TPH2.

3.3.4 Ubiquitination of TPH2 could occur in the absence of lysine residues

As previously mentioned, TPH2 has thirty-five lysine residues found along its amino acid sequence. Although site-directed

mutagenesis could be used to study the roles of each lysine residue in TPH2 ubiquitination, individual or combinational lysine-to-arginine substitutions could prove to be time-consuming. Furthermore, there have been earlier indications that multiple ubiquitination sites were present on TPH2, and the removal of a single lysine residue could result in compensation of its function by other non-substituted lysine residues. As such, replacing all thirty-five lysine residues with arginine could facilitate the determination of the role of these lysine residues in TPH2 ubiquitination. A lysine-less mutant TPH2 with all thirty-five lysine residues substituted with arginine was generated, and the HA-tag was appended to its N-terminus (HA-TPH2-KR490, Figure 3.6A). When overexpressed in PC12 cells, HA-TPH2-KR490 was found to have lower levels of expression, as well as migrate at a faster rate on SDS-PAGE, compared to wild type TPH2 (Figure 3.6, B and C). However, FLAG-Ub also co-immunoprecipitated with HA-TPH2-KR490, when both constructs were transiently expressed in MG132 treated PC12 cells (Figure 3.6D), which suggested that ubiquitination could occur on TPH2 even in the absence of lysine residues.

3.4 DISCUSSION

In this study, we investigated the mechanism of TPH2 degradation that could contribute to its instability *in vitro*, as well as search for sites on TPH2 that play a role in protein turnover. Similar to

TABLE 3.2. Predicted ubiquitination sites on TPH2

Lysine Residue Position	Score
UbPred Prediction	
38	0.88
54	0.77
63	0.87
183	0.70
BDM-PUB Prediction	
43	1.45
47	2.39
50	2.13
54	4.35
63	1.93
98	1.80
183	1.27
388	1.28
394	1.62
398	2.41
429	1.20

Two predictors were used for predication of ubiquitination sites on TPH2. Algorithms and threshold scores differed depending on the predictor used. UbPred identified 4 potential ubiquitination sites, with medium (0.69 ≤ Score ≤ 0.84) and high confidence (0.84 ≤ Score ≤ 1.00). BDM-PUB prediction identified 11 potential ubiquitination sites when performed at medium (1.00 ≤ Score) to high specificity (2.70 ≤ Score).

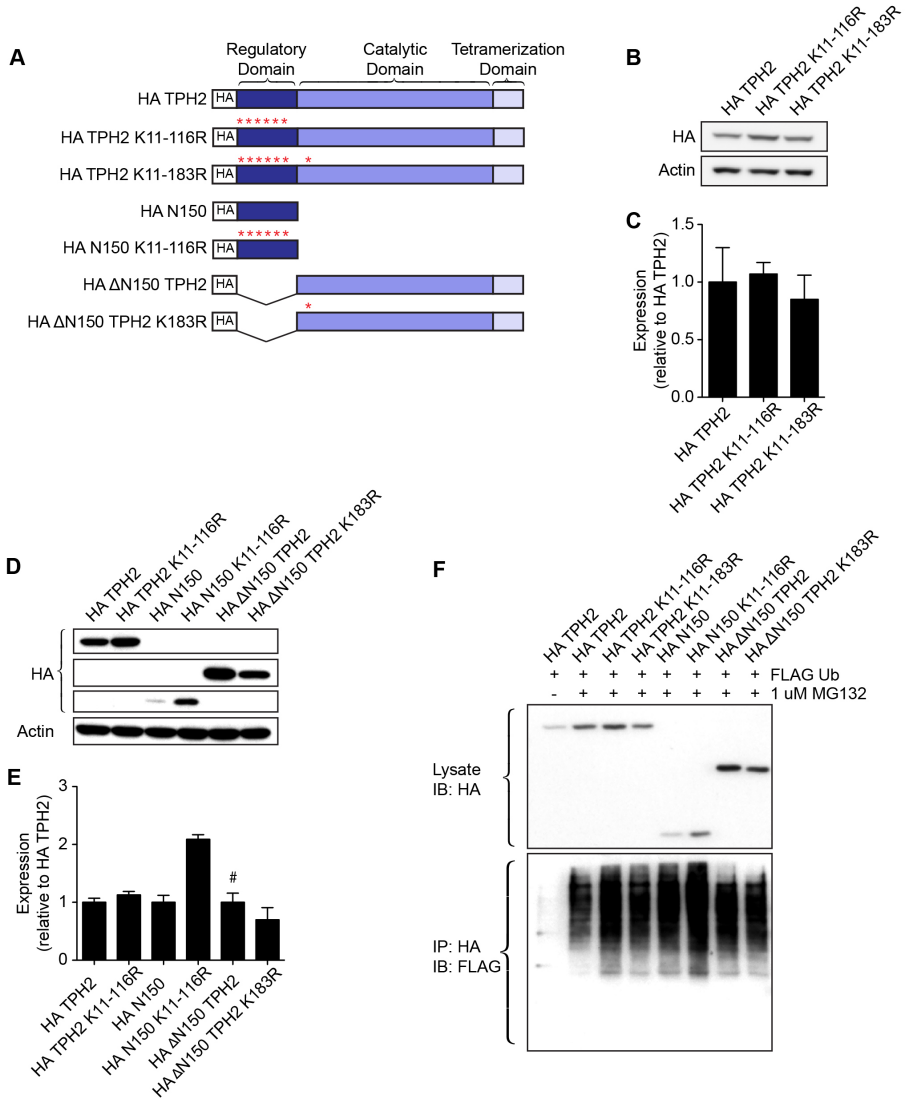

FIGURE 3.5. Non-conserved N-terminal lysines and predicted K183 did not affect ubiquitination of TPH2 domains. A, Lysine to arginine mutagenesis of K183 (K183R) was incorporated into existing HA-tagged K11-116R construct to generate HA-TPH2-K11-183R. All seven lysine to arginine substitutions were also introduced at their respective positions on HA-N150 and HA-ΔN150-TPH2 to generate HA-N150-K11-116R and HA-ΔN150-TPH2-K183R, respectively. B and D, All mutagenesis as well as wild type HA-tagged constructs were transiently expressed in PC12 cells, and expression levels were determined by Western blot. C and E, Quantification of expression levels of all mutagenesis constructs when overexpressed in PC12 cells. Expression levels were normalized to actin, and represented as a ratio to protein expression of wild type TPH2; #, $p < 0.05$ when compared to HA-N150-K11-116R. Data are means ± S.E. (n=3). F, All mutagenesis constructs were also co-expressed with FLAG-Ub in PC12 cells. In the presence of MG132, all transiently expressed proteins were observed to co-immunoprecipitate with FLAG-Ub, indicating that K11, K38, K43, K54, K63, K116 and K183 are not sufficient to cause TPH2 ubiquitination.

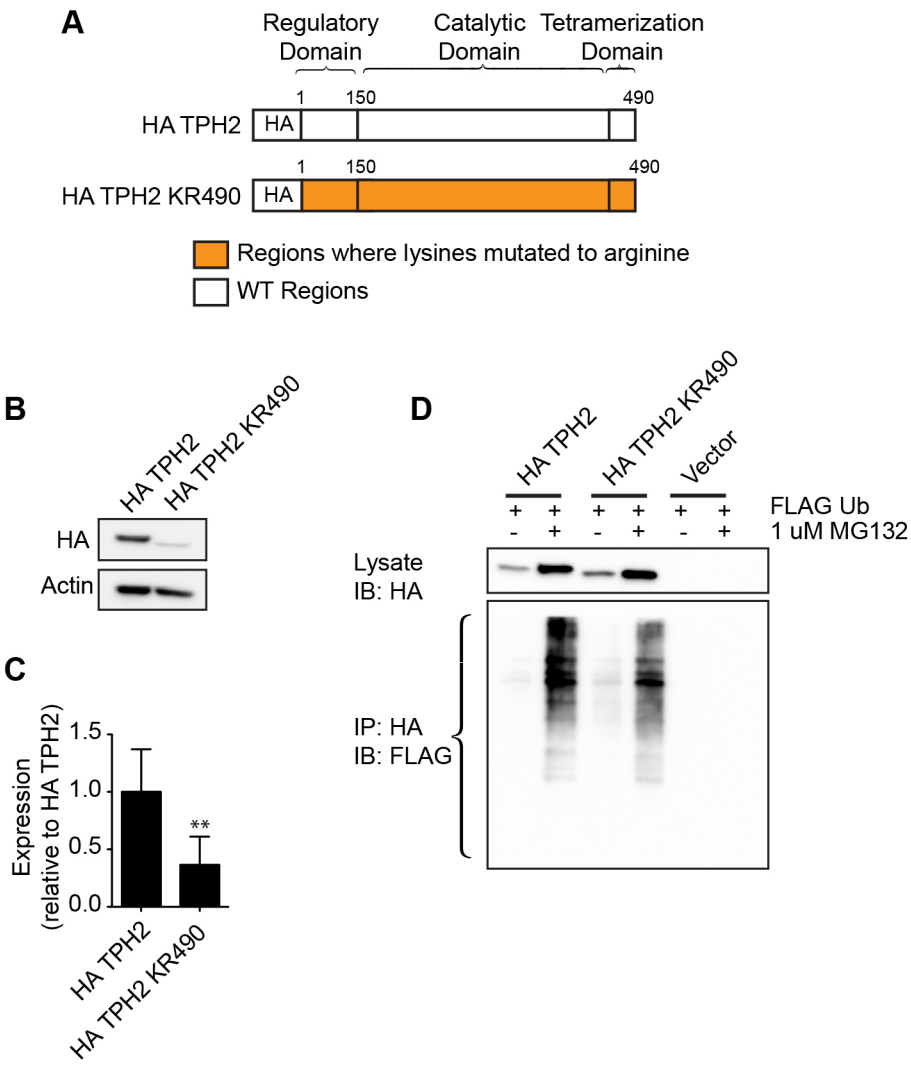

FIGURE 3.6. Lysine-less TPH2 could still conjugate to ubiquitin *in vitro*. A, Lysine-less TPH2, with all thirty-five lysines replaced with arginine residues, was synthetically generated and fused with a HA-tag at the N-terminus to construct HA-TPH2-KR490. B, Both wild type HA-TPH2 and HA-TPH2-KR490 were transiently overexpressed in PC12 cells. Expression levels were then examined using Western blot. C, Densitometric quantification of expression levels of HA-TPH2 and HA-TPH2-KR490 when overexpressed in PC12 cells. Expression levels were normalized to actin, and represented as a ratio to protein expression of wild type HA-TPH2; **, $p < 0.05$ compared to expression levels of HA-TPH2. Data are means ± S.E. (n=3). D, Wild type HA-TPH2 and HA-TPH2-KR490 were also co-expressed with FLAG-Ub in PC12 cells, which were then treated with MG132. IP and Western blot showed that HA-TPH2-KR490 could still associate with FLAG-Ub, despite the absence of lysine residues.

the previous chapter, PC12 cells were employed for the present study because they possessed neural characteristics, and also provided an efficient overexpression system for examining the degree of ubiquitination of TPH2 and its mutants under physiological conditions. We have showed that by inhibiting proteasomal degradation using MG132, TPH2 expression was significantly increased in PC12 cells that were stably or transiently expressing HA-TPH2. This is also the first report demonstrating that TPH2 could be heavily ubiquitinated. In contrast to earlier reports, little ubiquitination was detected on TPH1, although the increase in protein expression levels were similar to that previously described (Kojima, Oguro et al. 2000). The varying observations could be due to the differences in cell lines and protein expression techniques used. In this study, HA- and FLAG-tagged constructs were overexpressed in cells, since effective monoclonal antibodies against TPH2 were unavailable. Ubiquitination of TPH1 could still be detected in cells treated with MG132, albeit only at longer exposures (data not shown). This in turn also indicated that TPH2 could undergo ubiquitination more readily than TPH1, thus resulting in increased instability *in vitro*.

Although TPH2 ubiquitination and the involvement of proteasomal degradation have been demonstrated, the underlying mechanisms that gave rise to the differences in stability and extents of ubiquitination between TPH1 and TPH2 were still unknown. We initially

hypothesized that the differences in amino acid sequences, especially in the regulatory domains of both isoforms, could contribute to the differential ubiquitination and turnover rates. However, when non-conserved lysine residues in the TPH2 regulatory domain were substituted to arginine, immunoprecipitation revealed that TPH2 was still ubiquitinated. Mutation of possible ubiquination sites predicted by prediction software (i.e. K183) also failed to abolish TPH2 ubiquitination. This could be attributed to the limitations of the prediction software. UbPred and BDM-PUB were the software readily available when prediction was performed. These predictors were based on limited data sets obtained from experimental studies as well as literature mining (Li, Gao et al. 2009; Radivojac, Vacic et al. 2010). In addition, UbPred was built with a focus on ubiquitinated proteins in yeast. While functional categories of over-ubiquitinated proteins in yeast and human were demonstrated to be similar (Radivojac, Vacic et al. 2010), the prediction software could still have limitations due to small datasets and lack of specific consensus sequences for ubiquitination. This appeared to be the case for K183, since HA-TPH2-K11-183R was still observed to be ubiquitinated.

Recently, we analyzed the TPH2 amino acid sequence again using hCKSAAP_UbSite (Chen, Zhou et al. 2013), a more updated predictor based on a dataset of ubiquitinated human proteins. Seven positive prediction sites were revealed, but these sites were predicted

with low confidence and only one predicted lysine was located within the TPH2 regulatory domain (data not shown). The seven lysine residues that we originally identified were not presented as positive predicted sites by hCKSAAP_UbSite, which further supported our observations that lysine to arginine mutations of these seven residues still resulted in TPH2 ubiquitination. Although the seven newly predicted lysine residues could be further examined by similar mutagenesis techniques, the results are likely to be inconclusive. This is because in addition to demonstrating that both the regulatory and catalytic domains of TPH2 could be ubiquitinated, we have also shown that a lysine-less TPH2 could still undergo ubiquitination. These observations in turn suggested that TPH2 could be ubiquitinated via alternative mechanisms (Figure 3.7).

Due to the absence of consensus sequences, protein ubiquitination sites can be diverse. Some proteins, like IκBα and receptor interacting protein kinase 1 (RIP1), are ubiquitinated through distinct lysine residues (Scherer, Brockman et al. 1995; Ea, Deng et al. 2006). On the other hand, c-Jun can be ubiquitinated on any lysine residue along its amino acid sequence (Treier, Staszewski et al. 1994). Other proteins, such as Gcn4 and yeast uracil permease Fur4p, may be ubiquitinated at specific lysine residues that are proximal to an internal PEST degradation signal (Kornitzer, Raboy et al. 1994; Marchal, Haguenauer-Tsapis et al. 2000). In addition to internal

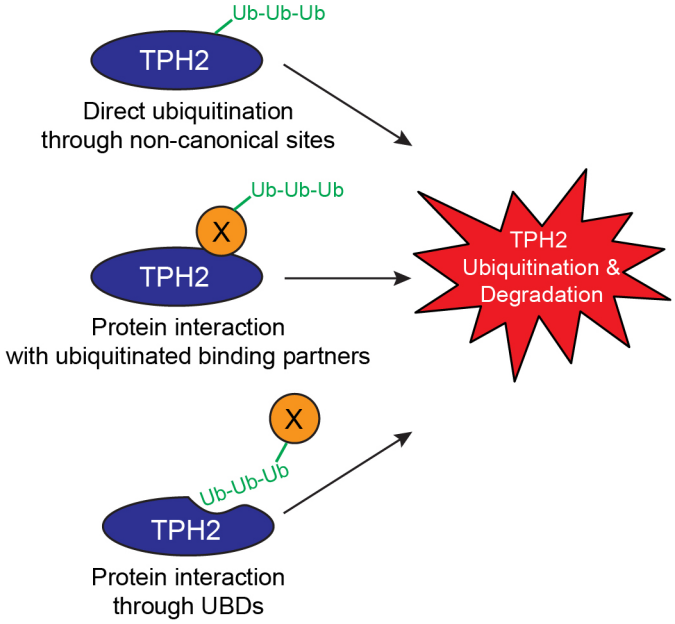

FIGURE 3.7. Possible mechanisms by which TPH2 can undergo ubiquitination and proteolysis. As lysine-less TPH2 was observed to be ubiquitinated, direct ubiquitination of TPH2 may occur through non-canonical sites, such as serine, threonine and cysteine residues. We also postulated that TPH2 can be ubiquitinated indirectly, through interaction with additional protein partners (X). TPH2 may interact with proteins that can be ubiquitinated themselves, allowing the protein complex to be broken down in the proteasome. Alternatively, TPH2 may contain UBDs that can also interact with ubiquitinated proteins, thereby facilitating proteasome degradation. Further studies will be required to examine these hypotheses.

lysines, ubiquitination has been shown to occur at non-canonical sites as well. Several proteins have been reported to undergo N-terminal ubiquitination, whereby ubiquitin is conjugated linearly to the α-NH_2 group of the N-terminal methionine (Breitschopf, Bengal et al. 1998; Aviel, Winberg et al. 2000; Bloom, Amador et al. ; Coulombe, Rodier et al. 2004; Kuo, den Besten et al. 2004; Li, Okamoto et al. 2009; Vosper, McDowell et al. 2009; McDowell, Kucerova et al. 2010). Lysine-less mutants of these proteins could still be ubiquitinated and efficiently broken down as a result of this novel mechanism. Increased protein stability was also detected when the N-terminal methionine residues of these proteins were fused with a 6x-myc tag (Breitschopf, Bengal et al. 1998; Aviel, Winberg et al. 2000; Bloom, Amador et al. 2003; Li, Okamoto et al. 2009; Vosper, McDowell et al. 2009). Similar observations were made in the case of TPH2, as we have shown in this study, where TPH2 ubiquitination did not require any lysine residues. Previous studies have also suggested that the addition of N-terminal fusion tags could improve TPH2 stability *in vitro* (McKinney, Knappskog et al. 2004; McKinney, Knappskog et al. 2005). However, the presence of HA-tags at the N-terminus of HA-TPH2 and HA-TPH2-KR490 could prevent ubiquitination of the N-terminal methionine. Protein expression levels of HA-TPH2-KR490 also unexpectedly decreased, unlike the unchanged protein levels observed in lysine-less mutants of N-terminal ubiquitinated proteins. Furthermore, HA-TPH2-KR490 appeared to migrate faster than wild type HA-TPH2 during

electrophoresis, indicating lower molecular weight. While the underlying mechanisms giving rise to these observations were unknown, the decreased expression and molecular weight of HA-TPH2-KR490 could imply the involvement of other post-translational modifications in regulating TPH2 expression. Nevertheless, further studies would be required to examine this possibility. Apart from the N-terminal methionine, recent studies have also reported ubiquitination of internal serine, threonine and cysteine residues (Cadwell and Coscoy 2005; Tait, de Vries et al. 2007; Wang, Herr et al. 2007; Vosper, McDowell et al. 2009; McDowell, Kucerova et al. 2010). Nevertheless, such cases are uncommon and also require ubiquitin to form atypical ester linkages with serine and threonine residues, or thioester linkages with cysteine residues. Further investigations would be required in order to establish whether these non-canonical sites are involved in TPH2 ubiquitination and subsequent degradation.

In addition to non-canonical ubiquitination sites, we postulated that the occurrence of TPH2 ubiquitination even in the absence of lysine residues could also be brought about protein-protein interactions. It is possible that TPH2 can interact with unknown protein partners that can undergo ubiquitination. As a result, ubiquitin will be detected to associate with TPH2 and the protein complex can subsequently be targeted for proteasomal degradation (Figure 3.7). Similarly, TPH2 may interact with other ubiquitinated proteins through ubiquitin binding

domains (UBDs), which are modular elements within proteins that can recognize and bind to ubiquitin non-covalently. UBDs can fold into a diversity of structural motifs, such as α-helices, zinc fingers, plekstrin homology folds, or ubiquitin conjugating domains, allowing interaction with ubiquitin to bring about functional outcomes like protein degradation (Hicke, Schubert et al. 2005; Dikic, Wakatsuki et al. 2009; Husnjak and Dikic 2012) (Figure 3.7). Taken together, the notion that additional interacting proteins are involved in TPH2 ubiquitination would imply that observations in this study were the result of indirect ubiquitination of TPH2, mediated through its association with ubiquitinated binding partners. Additional studies will be required in order to further explore these hypotheses, as well as identify any interacting partners and their roles in regulating TPH2 ubiquitination.

Earlier studies have reported that aromatic amino acid hydroxylases, such as TPH1, tyrosine hydroxylase and phenylalanine hydroxylase (TH and PAH) could be ubiquitinated (Doskeland and Flatmark 1996; Kojima, Oguro et al. 2000; Doskeland and Flatmark 2001; Doskeland and Flatmark 2002). However, ubiquitination sites have yet to be identified, although residues within the catalytic domain of PAH have been indicated to undergo ubiquitination (Doskeland and Flatmark 2001). Reports have also indicated that TPH1 and TH phosphorylation could trigger proteasomal degradation, suggesting that ubiquitination may be regulated by additional modifications (Iida,

Sawabe et al. 2002; Kawahata, Tokuoka et al. 2009; Nakashima, Mori et al. 2011). Nevertheless, the mechanisms by which phosphorylation could bring about TPH2 ubiquitination are still unknown. It is possible that phosphorylation can trigger conformational changes in TPH2, which may also improve its interaction with other protein partners that can bring about ubiquitination. However, this hypothesis still remains to be proven. While the findings of this study did not conclusively identify any ubiquitination sites on TPH2, the results have suggested that the difference in extent of ubiquitination between TPH1 and TPH2 is likely due to factors beyond the N-terminal regulatory domains alone. Further studies would be required to determine these regulatory factors and how they can give rise to the differential stabilities of TPH1 and TPH2.

As TPH2 plays a crucial role in the production of brain serotonin, mutations leading to decreased TPH2 activity or stability have been implicated in a wide range of neuropsychiatric disorders (Zhang, Gainetdinov et al. 2005; Carkaci-Salli, Salli et al. 2014). Taking into consideration that TPH2 is less stable and experiences a faster turnover rate as compared to TPH1, studying the TPH2 degradation pathway and the regulatory factors involved may provide valuable insights to the development of new therapeutic interventions. The identification of ubiquitination sites or domains on TPH2 could thus provide a foundation to further the understanding of TPH2 proteolysis,

as well as the differences in stability observed between TPH1 and TPH2.

4. DISCUSSION AND FUTURE DIRECTIONS

4.1 DISCUSSION

Since the discovery of TPH2 as the neuron-specific isoform of the 5-HT synthesizing enzyme within the central nervous system (CNS), various studies have been performed on both molecular and systemic levels, to further understand regulation of this enzyme. Given that 5-HT can influence mood, sleep, learning and neural development, amongst others, dysregulation of brain 5-HT homeostasis can bring about detrimental effects. Indeed, disruption of serotonin signaling has been implicated in various neuropsychiatric disorders, such as depression, bipolar disorder, anxiety and schizophrenia (Lucki 1998; Zhang, Gainetdinov et al. 2005; Lin, Chao et al. 2007; Zhang, Li et al. 2011; Berger, Weber et al. 2012).

Recent studies focused only on investigating transcriptional regulation of TPH2, and there is still a lack of studies pertaining to other regulatory factors that control TPH2 protein levels. Apart from the stabilizing functions of post-translational phosphorylation and 14-3-3 proteins (Murphy, Zhang et al. 2008; Winge, McKinney et al. 2008), mechanisms resulting in the instability and degradation of TPH2 are still not well understood. Until now, it is only known that the N-terminal regulatory domain confers a destabilizing effect on TPH2 expression and activity, and that the formation of disulphide linkages could cause

TPH2 to misfold, inhibiting its catalytic activity and resulting in aggregate formation (Carkaci-Salli, Flanagan et al. 2006; Kuhn, Sykes et al. 2011). Therefore, the current work sought to elucidate regulatory mechanisms underlying this instability of TPH2. Specifically, we studied potential protein-protein interactions that can affect the stability of TPH2 *in vitro*, and also examined the role of ubiquitination and proteasomal degradation in TPH2 protein turnover. In both cases, our findings have revealed previously undiscovered aspects of TPH2 regulation, which can form the basis of future studies.

Improving the understanding of the regulation of TPH2 expression and activity at the protein level may be important in the development of future therapeutic drugs, particularly in the treatment of neuropsychiatric illnesses. Currently available antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) like fluoxetine, citalopram and paroxetine, act by blocking the serotonin transporter (SERT), which is present in both CNS and peripheral tissues. This may cause unanticipated side effects, arising as a result of SSRI action in the periphery. In comparison, TPH2 specifically controls synthesis of brain 5-HT, making it a therapeutic target that can potentially produce fewer side effects. Furthermore, TPH2 polymorphisms giving rise to missense mutations, such as P447R, have been shown to affect response to citalopram treatment (Cervo, Canetta et al. 2005). A recent study has also reported that chronic fluoxetine and paroxetine

treatment can worsen brain 5-HT deficiency in R439H Tph2 KI mice (Siesser, Sachs et al. 2013). Thus, a better understanding of TPH2 regulation would be important to resolve these different responses to SSRI treatment. Characterization of TPH2 protein regulation can also reveal additional regulatory factors and protein interacting partners that may serve as novel therapeutic targets in neuropsychiatric treatment.

4.2 FUTURE DIRECTIONS

4.2.1 Heat shock proteins and other interacting partners of TPH2

The identification of novel protein interacting partners of TPH2 can give us new insights into the regulatory pathways involved in TPH2 regulation. Differences in strengths of interactions as well as the identities of interacting partners associating with TPH1 and TPH2 can also allow us to infer the regulatory mechanisms giving rise to the different characteristics of both isoforms. The present study has demonstrated that although both TPH1 and TPH2 interact with HSP70, the weaker interaction detected between TPH2 and HSP70 could result in increased instability, as we have also shown that HSP70 has a stabilizing effect on TPH2 protein expression and activity. Nevertheless, in order to fully understand the role of HSP70 in relation to TPH2, and even TPH1, additional studies can be performed to support our current findings.

As our co-immunoprecipitation (IP) results indicated that HSP70 could interact more strongly with TPH1 and weaker with TPH2, surface plasmon resonance (SPR) can be used to further support these observations. SPR allows for the measurement of binding affinity between unlabeled protein interacting partners. This will in turn circumvent the use of HA-tagged proteins in the current study, and also provides a more quantitative measure on strength of interaction between the binding partners. Our co-IP studies have also established that the first 10 amino acids of TPH1 were primarily involved in efficient HSP70 interaction. However, the underlying mechanisms by which this non-conserved stretch of amino acid residues gives rise to the differential extents of HSP70 interaction between TPH1 and TPH2 is still not understood. By using site-directed mutagenesis, co-IP and even SPR, the roles of these 10 amino acids on TPH1, and the corresponding residues on TPH2, can be further examined with respect to HSP70 interaction.

We have demonstrated that the effects of HSP70 knockdown on TPH2 expression could be rescued by heat shock treatment followed by a short recovery period, which brought about an increase in endogenous HSP70. Nevertheless, overexpression of exogenous HSP70 will be a more specific approach for rescuing HSP70 knockdown and should be performed to further reinforce the current observations. However, given that HSP70 plays a role in both protein

stabilization and proteolysis, transient overexpression of exogenous HSP70 may disrupt the relative stoichiometries of HSP70 and TPH2 within the cells. Thus, optimization is first required to determine the amount of exogenous HSP70 to be transiently overexpressed in order to rescue the effects of HSP70 knockdown.

On a similar note, increase in protein expression levels was not observed when exogenous HSP70 was transiently overexpressed in PC12 cells stably expressing HA-TPH2 (data not shown). We postulated that this could be due to the saturation of HSP70 binding sites, and therefore increasing HSP70 levels transiently would not affect TPH2 expression or stability further. Furthermore, transient overexpression of exogenous HSP70 may also disrupt the physiological levels of HSP70 within the cells, which in turn can lead to unexpected effects (as described above, and in (Roy, Li et al. 2005)). In order to overcome this, a stable knockdown or knockout cell line can be employed, such as PC12 cells with a stable knockdown of HSP70, or HSP70-/- mouse embryonic fibroblasts (MEFs). Overexpression of exogenous HSP70 in these cell lines will rescue the effects of lowered HSP70 levels on TPH2 expression and activity more effectively, although optimization of the amount of exogenous genes introduced may still be required.

As the scope of the current work was limited to *in vitro* studies, the role of HSP70 in relation to TPH2 activity and expression should also be investigated using *in vivo* models, such as knockout or knock-in mice. TPH2 expression and activity can be analyzed in brain tissue of HSP70 knockout mice, using Western blot and HPLC, to substantiate our *in vitro* data. In addition, potential behavioral changes can be assessed in these HSP70 knockout mice, which may result from changes in the levels and stability of TPH2 within the brain. Similarly, HSP70 expression can be induced in TPH2 knock-in mice, and changes in TPH2 levels, activity and effects on mouse behavior can then be investigated.

Finally, the identification of HSP70 as a novel interacting partner of TPH1 and TPH2 also indicated that there could still be other potential interacting proteins that are yet to be uncovered. As the current study only identified interacting partners of approximately 70 kDa, additional methods that do not impose a limit on molecular weight can be used to detect interacting proteins from whole cell lysate. Co-IP and 2D electrophoresis or 2D-difference gel electrophoresis (2D-DIGE) can be employed, which will separate interacting partners effectively. In the case of 2D-DIGE, comparisons between protein partners of TPH1 and TPH2 can also be made. MALDI-TOF/TOF MS can then be used to analyze and identify interacting partners. Alternatively, whole IP fractions can also be directly subjected to trypsin digestion and MALDI-

TOF/TOF MS identification. The detection of distinct interacting partners between TPH1 and TPH2 may indicate possible regulatory differences between the two isoforms, and thus can be further investigated. In this study, we have also identified potential interacting partners of TPH1 in HSP70B', HSC70 and HSPA1L. Although no further work was carried out on them, the interaction of the three identified proteins with TPH1 can be validated using co-IP and antibodies against endogenous proteins. RNA interference can also be employed to study the functions of these potential partners with respect to TPH1 regulation.

4.2.2 Ubiquitination and TPH2 regulation

Understanding the mechanisms involved in TPH2 degradation and proteolysis may allow us to further appreciate the underlying causes that give rise to the instability of the enzyme. While the regulatory domain of TPH2 has been shown to play a role in destabilizing protein expression and activity, the subsequent pathway by which TPH2 is broken down, and whether the regulatory domain is involved, is still not well understood. We have demonstrated that TPH2 could be heavily ubiquitinated and degraded via the proteasome. However, ubiquitination sites on TPH2 still remain elusive. In this section, we propose additional experiments that can be carried out to identify residues that can be modified by ubiquitination, which in turn

may allow us to further elucidate the degradation pathway and regulators involved.

Non-canonical ubiquitination has been implicated in TPH2 degradation, since lysine-less TPH2 was observed to still be ubiquitinated. As such, the roles of atypical ubiquitination sites, such as serines, threonines and cysteines, should be further investigated. To examine the possibility of atypical ubiquitination sites, serine-less, threonine-less and cysteine-less TPH2 mutants can be generated using mutagenesis. Co-expression with FLAG-Ub and IP may then be used to assess the possibility of ubiquitination on these mutants. In addition, the role of phosphorylation in TPH2 ubiquitination can be further explored. To this end, mutagenesis of known TPH2 phosphorylation sites, like S19 and S104, can be carried out, and the stability and ubiquitination of the mutants can be assessed.

In this study, we utilized IP to detect ubiquitination sites, indicated by the association of exogenous FLAG-Ub with HA-tagged TPH2 and its mutants. Nevertheless, this approach requires generating numerous TPH2 mutants in order to narrow down potential ubiquitination sites, which can be fairly tedious and time-consuming. Methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) are increasingly gaining popularity due to the speed and efficiency in which ubiquitinated residues can be identified (Peng,

Schwartz et al. 2003; Coulombe, Rodier et al. 2004; Anania, Bustos et al. 2013). Thus, identification of ubiquitination sites is also possible using this approach. Furthermore, mass spectrometry can be employed to address the possibility of additional protein partners interacting with TPH2. Using co-IP, 2D-DIGE and MALDI-TOF/TOF MS, any potential interacting protein partners of HA-TPH2 during inhibition of proteasomal degradation may be identified and further studied.

Lastly, the observation that TPH2 was ubiquitinated also indicates that an E1-E2-E3 ubiquitination cascade is catalyzing ubiquitin linkage to TPH2. As E3 ubiquitin ligases are able to recognize the specific target substrate(s) and catalyze the attachment of ubiquitin, identifying the E3 ligase involved in TPH2 ubiquitination may reveal a crucial regulator of TPH2 stability as well. Co-IP, MALDI-TOF/TOF MS, and yeast-two-hybrid are possible means by which the TPH2-specific E3 ligase can be determined. Alternatively, knockdown of known E3 ligases using RNA interference may also be carried out *in vitro* to assess their effects on TPH2 expression.

4.2.3 Conclusion

The results presented in this study have revealed a new TPH2-interacting protein, as well as demonstrated the role of ubiquitination in TPH2 degradation. Our work has provided more insights to the

regulatory mechanisms underlying the instability of TPH2 at the protein level, which can be further supported by the additional studies illustrated in this chapter. This in turn can provide the basis for additional research that may further our understanding as to how the brain serotonergic system is being regulated at a molecular level. The new knowledge will also be able to contribute to the development of new therapeutics against neuropsychiatric conditions involving 5-HT dysregulation.

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