Function and regulation of phenylalanine and tyrosine hydroxylases from human and *Caenorhabditis elegans*

With focus in evolutionary aspects and development of new therapies

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A mis padres

y

a mi abuela

To my parents

and

my grandmother

Don't give up, please don't give way,
Even if the cold burns,
Even if fear bites,
Even if the sun sets,
And the wind goes silent,
There is still fire in your soul
There is still life in your dreams.

(Don´t give up, Mario Benedetti)

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Abstract

The family of the aromatic amino acid hydroxylases (AAAH) is well studied in mammals. It includes four members, i.e. phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and the tryptophan hydroxylases (TPH1 and 2). These enzymes share important features, such as domain organization, three dimensional structure and mechanism of the reaction. The AAAH have important functions and are related to genetic human diseases. PAH, expressed in liver, is in charge of L-Phe catabolism from the diet and mutations in the *PAH* gene lead to phenylketonuria (PKU), a paradigm for genetic metabolic diseases. TH and the TPHs are enzymes of the neuroendocrine system, that carry out the rate limiting steps in the synthesis of neurotransmitters and hormones, i.e. catecholamines (TH) and serotonin and melatonin (TPHs). Mutations in *TH* and the *TPHs* genes are also involved in important neurological diseases and disorders, such as some forms of dystonia and parkinsonism in the case of TH and mood disorders in TPH.

The nematode *Caenorhabditis elegan*s is a model organism widely used in biology. We here present the expression and characterization of two AAAH from the nematode, PAH and TH, in order to get insights into evolution of structure, function and regulation in this enzymatic family. In the case of PAH we found functional and molecular similarities between *C. elegans* PAH (cePAH) and human PAH (hPAH), although they display important differences in enzymatic activity regulation, especially regarding the regulation exerted by the substrate, L-Phe. Both the preactivation and the positive cooperativity induced by the substrate on mammalian PAHs were absent in cePAH. *In vivo* experiments with knock-out worms bearing a deletion in the *pah* gene (*pah-1*) demonstrated that cePAH is involved in the synthesis of a melanin-like compound that localizes in the cuticle. The study of the recombinant TH from *C. elegans* (ceTH) in comparison with human TH (hTH) also revealed important differences at the level of short-term activity regulation. Basic regulatory mechanisms for hTH, such as substrate inhibition and feed-back inhibition by the end catecholamine products, appear to be absent in ceTH, suggesting a less tight regulation of enzymatic activity in the worm. But interestingly, ceTH was effectively phosphorylated by cAMP-dependent protein kinase (PKA) at Ser35, though this modification did not translate into activation of the enzyme in synergy with the feed-back inhibition by catecholamines, as

it is the case for phosphorylation of hTH at the equivalent Ser40. We hypothesised that phosphorylation of ceTH could regulate the interaction with other proteins and/or control subcellular localization.

Supplementation with BH_4 has been recently established as a therapeutic intervention for PKU. A main effect of the cofactor is the stabilisation of misfolded PKU mutants, and BH_4 appears to function as a natural chaperone molecule. Since BH_4 is a shared cofactor by all the AAAH we set to investigate the effect of BH_4 supplementation on rat brain TH. Higher doses of BH_4 than those currently used for the treatment of PKU were needed to increase the cofactor concentration in brain, most probably due to the selectivity of the blood-brain-barrier (BBB). This indicates that the current treatments using lower doses of BH_4 (up to 20 mg/kg/day) are not expected to affect neuronal TH and TPH2. An increment of total TH protein and activity was measured in the brains of wild-type (wt) mice upon treatment with 100 mg BH_4/kg/day, suggesting that BH_4 also functions as a natural chaperone in the case of TH. In agreement with these effects, *in vitro* experiments also showed the capability of BH_4 to stabilise TH.

Finally, the screening of chemical libraries of small organic compounds is arising as a promising tool to find specific stabilisers of proteins (i.e. pharmacological chaperones). In the case of PAH, four stabilising compounds (compounds I-IV) were found in a previous study, revealing their potential as therapeutic pharmacological chaperones for PKU. As in the case of BH_4, it was interesting to study the effect of these four molecules upon neuronal TH and TPH2. We found that compound III stabilized the three AAAH investigated, whereas the other compounds exerted different enzyme specific effects. *In vivo* studies with supplemented mice revealed the potential of compound III to treat TH-associated diseases. These results are important not only for the development of new specific therapies, but also to unravel enzyme specific/non specific ligand binding in the AAAH family.

List of publications

I. Calvo A.C., A.L. Pey, Ying M., Loer C.M. and Martinez A. (2008) Anabolic function of phenylalanine hydroxylase in *Caenorhabditis elegans*. *FASEB J.* **22**: 3046-58.

II. Calvo A.C., Pey A.L., Miranda-Vizuete A. and Martinez A. (2010) Cloning and biochemical characterization of the enzyme tyrosine hydroxylase from the nematode *Caenorhabditis elegans. Manuscript.*

III. Thony B.*, Calvo A.C.*, Scherer T., Svebak R.M., Haavik J., Blau N. and Martinez A. (2008) Tetrahydrobiopterin shows chaperone activity for tyrosine hydroxylase. *J. Neurochem*. **106**: 672-81.
*the first two authors have equally contributed to this work

IV. Calvo A.C., Scherer T., Pey A.L., Ying M., Winge I., McKinney J., Haavik J., Thony B. and Martinez A. (2010) Effect of pharmacological chaperones on brain tyrosine hydroxylase and tryptophan hydrolylase 2. *J. Neurochem. In Press.*

Related publications not included in the PhD thesis

V. Pey A.L., Martinez A., Charubala R., Maitland D.J., Teigen K., Calvo A., Pfleiderer W., Wood J.M. and Schallreuter K.U. (2006) Specific interaction of the distereomers 7(R) and 7(S)-tetrahydrobiopterin with phenylalanine hydroxylase- Implications for understanding primapterinuria and vitiligo. *FASEB J.* **20**: 2130-2.

VI. Martinez A., Calvo A.C., Teigen K. and Pey A.L. (2008) Rescuing proteins of low kinetic stability by chaperones and natural ligands; phenylketonuria, a case study. *Prog. Mol. Biol. Transl. Sci.* **83**: 89-134.

Abbreviations

4a-OH-BH_4	tetrahydropterin-4a-carbinolamine
AAAH	aromatic amino acid hydroxylases
AADC	aromatic amino acid decarboxylase
BBB	blood-brain-barrier
BH_4	tetrahydrobiopterin
DHPR	dihydropteridine reductase
DRD	dopa responsive dystonia
GFP	green fluorescence protein
GTPCH	GTP cyclohydrolase
h	Hill coefficient
HTP	high throughput screening
L-DOPA	L-3,4-dihydroxyphenylalanine
L-Phe	L-phenylalanine
L-Trp	L-tryptophan
L-Tyr	L-tyrosine
NOS	nitric oxide synthase
PAH	phenylalanine hydroxylase
PCD	pterin-4a-carbinolamine dehydratase
PKA	cAMP-dependent protein kinase A
PKU	phenylketonuria
PTPS	pyruvoyl-tetrahydrobiopterin synthase
QCS	quality control system
RTS	rapid transcription translation system
SOD	super oxide dismutase
SR	sepiapterin reductase
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
UPP	ubiquitin-proteasome pathway
wt	wild type

1. General introduction

1.1. The family of aromatic amino acid hydroxylases (AAAH)

The family of the aromatic amino acid hydroxylases (AAAH), responsible for the metabolism of aromatic amino acids, includes phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and the tryptophan hydroxylases (TPH1 and 2). These enzymes share many properties, such as domain organization, high similarity at the amino acid sequence level, and requirement for iron and a cofactor, tetrahydrobiopterin (BH_4), for catalytic activity (for reviews about the AAAH family see (Fitzpatrick, 1999; Teigen et al., 2007)). On the other hand, they have some differing characteristics, as cellular localization, regulation or substrate specificity, key properties to understand functional and evolutionary aspects.

In the following paragraphs we will concentrate on the characteristics of TH and PAH, the AAAH mainly studied in this work. However, some basic properties of the TPHs will be also introduced at the end of this section.

1.1.1. The reaction catalysed by PAH and TH

The AAAH catalyse a hydroxylation reaction dependent on the specific amino acid substrate, the cofactor BH_4, molecular oxygen and a non-heme iron molecule at the catalytic site. It is generally accepted that the hydroxylation reaction mechanism is similar for all the AAAH (Fitzpatrick, 2003).

1.1.1.1. PAH reaction

PAH is present mainly in liver, but it is also abundant in kidney (Kaufman, 1971; Moller et al., 2000), and hydroxylates the amino acid L-phenylalanine (L-Phe) in the para position into L-tyrosine (L-Tyr) (Figure 1). In mammals, L-Phe is an essential amino acid (Kalhan and Bier, 2008) that we have to take in the diet. Accumulation of high levels of L-Phe in brain is highly toxic, underlining the importance of PAH. Its implication in the effective degradation of L-Phe from the diet identifies PAH as a "catabolic enzyme", in contrast to TH and TPH, considered "anabolic enzymes". But

PAH has also an anabolic face, since it supplies L-Tyr for the organism, converting this amino acid into a non-essential one in mammals (Kilani et al., 1995).

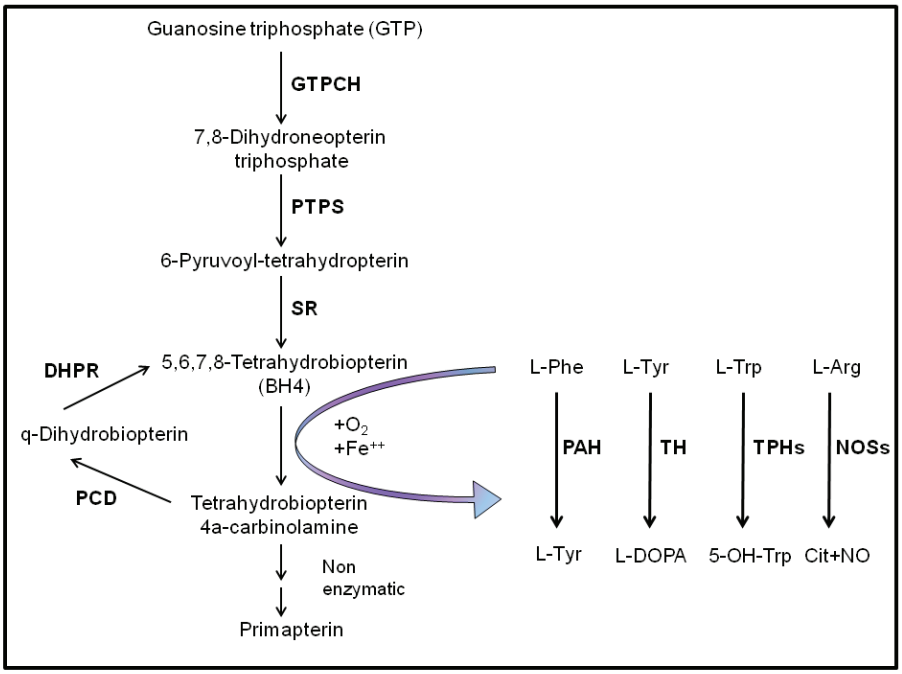


Figure 1. The BH_4 synthesis and recycling pathways (left part of the picture) and the reactions catalysed by PAH, TH, the TPHs and the NOSs (right part of the picture). Enzymatic abbreviations used in the picture: GTPCH (GTP cyclohydrolase), PTPS (pyruvoyl-tetrahydrobiopterin synthase), SR (sepiapterin reductase), PCD (pterin-4a-carbinolamine dehydratase), DHPR (dihydropteridine reductase), PAH (phenylalanine hydroxylase), TH (tyrosine hydroxylase), TPHs (tryptophan hydroxylases) and NOSs (nitric oxide synthases).

1.1.1.2. TH reaction

TH is implicated in the synthesis of neurotransmitters and hormones in the neuroendocrine system, mainly in brain and in chromaffin cells of the adrenal medulla. TH hydroxylates L-Tyr into L-dihydroxyphenylalanine (L-DOPA), the first and rate-limiting step in the synthesis of catecholamines (Figures 1 and 2). L-DOPA is then converted into dopamine through the reaction catalysed by aromatic amino acid decarboxylase (AADC). Further reactions will produce the neurotransmitters adrenaline and noradrenaline (Figure 2).

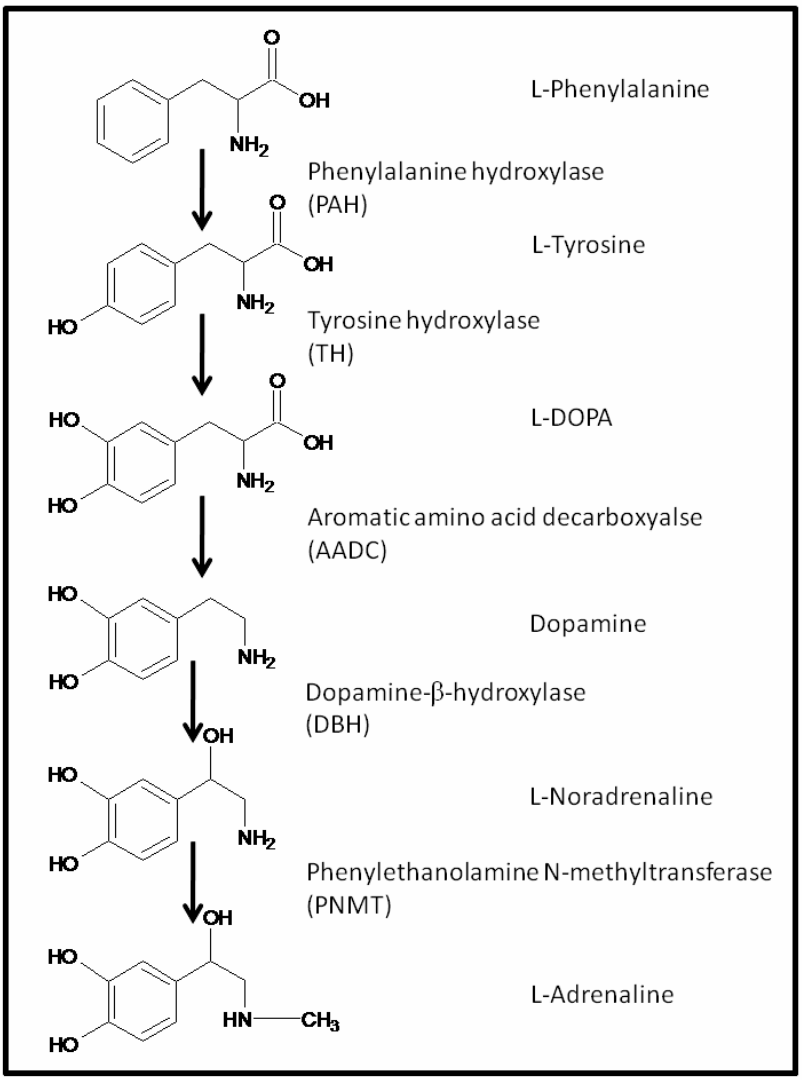


Figure 2. The pathway for the synthesis of catecholamines (dopamine, adrenaline and noradrenaline) from its precursor phenylalanine.

1.1.1.3. Reaction mechanism

Several studies have been carried out to study the kinetic mechanism of the AAAH, and it is still not clear how the reaction takes place. In the case of TH, Fitzpatrick reported an ordered mechanism of binding with the tetrahydropterin binding first, followed by oxygen and finally the amino acid substrate (Fitzpatrick, 1991). In the

case of PAH, studies with some of the bacterial forms of the enzyme (Pember et al., 1987; Volner et al., 2003) indicate that the pterin, amino acid substrate and oxygen bind in consecutive order. The mechanism of eukaryotic PAH remains controversial. A common feature for all the AAAH seems to be the need of the pterin, amino acid substrate and O_2 bound to the enzymes before the catalysis occurs (for a review see (Fitzpatrick, 2003)). During the catalytic cycle BH_4 is oxidized to tetrahydrobiopterin-4a-carbinolamine (4a-OH-BH_4) and the dioxygen atoms are in fact incorporated both into the substrate and the pterin cofactor. A high-spin Fe^{IV} species have been proposed as the hydroxylating intermediates and Fe^{IV}=O species have been directly observed in catalytically relevant complexes of TH (Eser et al., 2007).

1.1.2. The cofactor tetrahydrobiopterin (BH_4)

The AAAH share the same cofactor in the reaction, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). BH_4 belongs to a family of compounds called pteridines, with multiple and important biological functions. Pteridines are based on a two ring structure containing a fused pyrimidine and a pyrazine ring and were first identified as yellow pigments purified from butterflies wings (Longo, 2009) (Figure 3). For reviews about the synthesis and functions of BH_4 see (Thony et al., 2000; Werner et al., 2003).

1.1.2.1. The BH_4 biosynthetic pathway

The biosynthesis of BH_4 proceeds by three metabolic reactions from the precursor GTP (Figure 1). The first reaction is catalysed by the enzyme GTP cyclohydrolase (GTPCH), the main regulatory point in the BH_4 synthesis pathway (Mori et al., 1997; Cai et al., 2002; Tatham et al., 2009). GTPCH shows a well conserved sequence along evolution, notably with respect to the residues implicated in pterin binding and catalysis. The main divergence at the N-terminal domain may reflect regulatory differences between species (McLean et al., 1990; Witter et al., 1996; Golderer et al., 2001)).

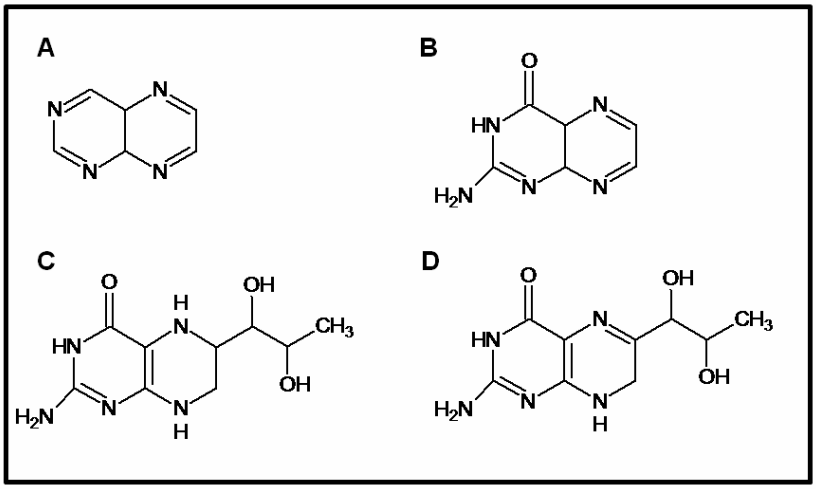


Figure 3. Pteridine structures**.** A) Pteridine; B) Pterin; C) 5,6,7,8-tetrahydrobiopterin (BH_4); D) 7,8-dihydrobiopterin (BH_2).

Pyruvoyl-tetrahydrobiopterin synthase (PTPS) is the second enzyme in the BH_4-synthesis pathway and it also constitutes an important regulatory step (Linscheid et al., 1998). Although there is not as much information available as for GTPCH, its conservation through species has also been demonstrated (Kim et al., 1996; Ben et al., 2003).

The last BH_4 biosynthetic enzyme is sepiapterin reductase (SR), which is also implicated in the "salvage route" of BH_4 synthesis (Auerbach et al., 1997). Evolutionary information between species for this enzyme is less abundant, compared with GTPCH and PTPS, but we can find homologues of SR in some invertebrate organisms (Seong et al., 2000; Meng et al., 2009).

1.1.2.2. Enzymes involved in the recycling of BH_4

BH_4 is usually referred to as a cofactor, but it is in fact a co-substrate that is modified during the catalysis, producing 4a-OH-BH_4, an oxidised form of the pterin. This compound has to be quickly catalyzed, since spontaneous non-enzymatic conversion of 4a-OH-BH_4 produces toxic metabolites (e.g, primapterin) (Curtius et al., 1990; Davis et al., 1991). 4a-OH-BH_4 is thus regenerated to BH_4 through two enzymatic

steps, catalysed by pterin-4a-carbinolamine dehydratase (PCD) and dihydropterin reductase (DHPR) (Thony et al., 2000).

1.1.2.3. Biological functions of BH_4

BH_4 has multiple functions that we could divide into two categories: enzymatic cofactor and cellular effector.

- Enzymatic cofactor: apart of its implication on the AAAH reactions (Figure 1) BH_4 is also the cofactor for the enzymes nitric oxide synthases (NOSs) and glycerol-ether mono-oxygenase (Taguchi and Armarego, 1998; Wei et al., 2003; Watschinger et al., 2009).
- Cellular functions: BH_4 has also several functions in the cell "per se", such as growing factor or protecting factor for nitric oxide (NO) in neurons (Tanaka et al., 1989; Anastasiadis et al., 1997; Werner et al., 2003). Moreover, BH_4 has also been assigned a neurotransmitter release-stimulating role, especially for dopamine and serotonin (Mataga et al., 1991).

1.1.3. Function and cellular localization of PAH and TH

1.1.3.1. Liver PAH and degradation of L-Phe from the diet

PAH is a cytosolic protein mainly found in liver but also in kidney (Kaufman, 1971; Moller et al., 2000). Although PAH is traditionally considered a catabolic enzyme, since the major function is the degradation of L-Phe from the diet, it is in fact a dual catabolic-anabolic enzyme because it also produces a supply of L-Tyr for the organism, converting this amino acid into a non-essential one (Hufton et al., 1995).

It seems that an alternative extra function for PAH in lower eukaryotes, together with TH and a group of enzymes called tyrosinases, is the synthesis of melanin and melanin-like compounds (Wiens et al., 1998; Johnson et al., 2003; Infanger et al., 2004; Leiros et al., 2007). Melanin has multiple functions; maybe one of the most important and characterized role is its implication in the immune system of invertebrate animals, such as *Drosophila melanogaster* or some species of mosquitoes (Muller et al., 1999; Leclerc and Reichhart, 2004; Cerenius et al., 2008). The role of PAH in melanin production in humans has also been suggested (Schallreuter et al., 2004; Schallreuter et

al., 2008). However, this function is not completely acknowledged, despite the fact that it has been shown that PAH is also expressed in the epidermis (Schallreuter et al., 2005).

PAH from *D. melanogaster* has been cloned and studied extensively (Bel et al., 1992; Silva et al., 1992), and immunological studies have detected the enzyme in the cuticular fraction of the fly, suggesting a role in formation and hardening of the cuticle (Silva et al., 1992). Since melanin is also present in the cuticle of *Drosophila* a role of PAH related to the synthesis of melanin is not unlikely (Wittkopp et al., 2002), taking into account that the product of the PAH reaction, L-Tyr, is the precursor in the melanin synthesis pathway.

1.1.3.2. Neuroendocrine TH and synthesis of catecholamines

In mammals TH is found in diverse tissues of the central and sympathetic nervous system: brain, adrenal medulla and other peripheral sympathetic neurons (Flatmark and Stevens, 1999; Fitzpatrick, 2000; Flatmark et al., 2002). Although TH is considered a cytosolic protein, a membranous fraction has also been reported (Kuhn et al., 1990; Thórólfsson et al., 2002). The extension or physiological significance of this fraction is unclear, but it has been proposed to be associated to the coupling of synthesis and storage of neurotransmitters in the synaptic vesicles at the synaptic cleft (Tsudzuki and Tsujita, 2004). An unexplored and interesting field is emerging in relation with the TH binding to the synaptic vesicle membrane (Cartier et al., 2010).

The main function of TH is the synthesis of catecholamines (Figure 2), functioning as hormones and neurotransmitters both in invertebrates and vertebrates. In relation with diseases such as Parkinson's disease or Dopa responsive dystonia (DRD), the control of locomotor functions -spontaneous and voluntary movements- arises as one of the most studied roles of TH (Laverty, 1978; Nishii et al., 1998). Lately, catecholamines have been involved in other cognitive and more complicated processes, such as behaviour, mood states or even learning (Kobayashi, 2001; Fernstrom and Fernstrom, 2007). The emergence of simpler organisms as animal models to study neurological functions and development, like *D. melanogaster* or *Caenorhabditis elegans*, opens the possibility of exploring these new roles of dopamine, adrenaline and noradrenaline (Tierney et al., 2003; Sanyal et al., 2004; Kindt et al., 2007).

TH is also implicated in the synthesis of melanin (Walter et al., 1996) providing a pool of L-DOPA used for the synthesis of melanin-like compounds, although the main suppliers for L-DOPA are the enzymes called tyrosinases (van Gelder et al., 1997; Halaouli et al., 2006).

1.1.4. Structure, domain composition, and molecular genetics of PAH and TH

All mammalian AAAH are oligomeric proteins, composed by four subunits. Each subunit contains three well differentiated domains: i) the N-terminal regulatory domain which varies in length depending on the enzyme, from around 100 residues in PAH to 160 in the largest isoform of human TH, i.e. TH4, ii) the central catalytic domain with around 280 residues and iii) C-terminal oligomerization domain containing 45-50 residues (Flatmark and Stevens, 1999) (Figure 4A and Appendix). TH, PAH and the TPHs are highly homologous proteins, reflecting the fact that all arise from a single locus through evolution (Ledley et al., 1985; Grenett et al., 1987; Siltberg-Liberles et al., 2008). Whereas the catalytic domain (also called catalytic core) (Fitzpatrick, 2000) is almost identical between the different proteins of the family, the regulatory domain shows a low degree of similarity, pointing to different regulatory mechanisms (Appendix).

The three dimensional crystal structure has been solved for a number of truncated forms of the enzymes (for a review see (Flatmark and Stevens, 1999)). Human PAH lacking the first 102 and the last 25 residues (Erlandsen et al., 1997) and rat TH lacking the first 155 residues (Goodwill et al., 1997), were the first structures solved for this family, and revealed the high structural similarity. At present we have access to more complete crystal structures, including the regulatory domain of rat PAH (Kobe et al., 1999). The structure for the full length tetrameric form has not been solved yet for any of the AAAH although composite structural models have been prepared (Flatmark and Stevens, 1999; McKinney et al., 2001; Teigen and Martínez, 2003) (Figure 4B).

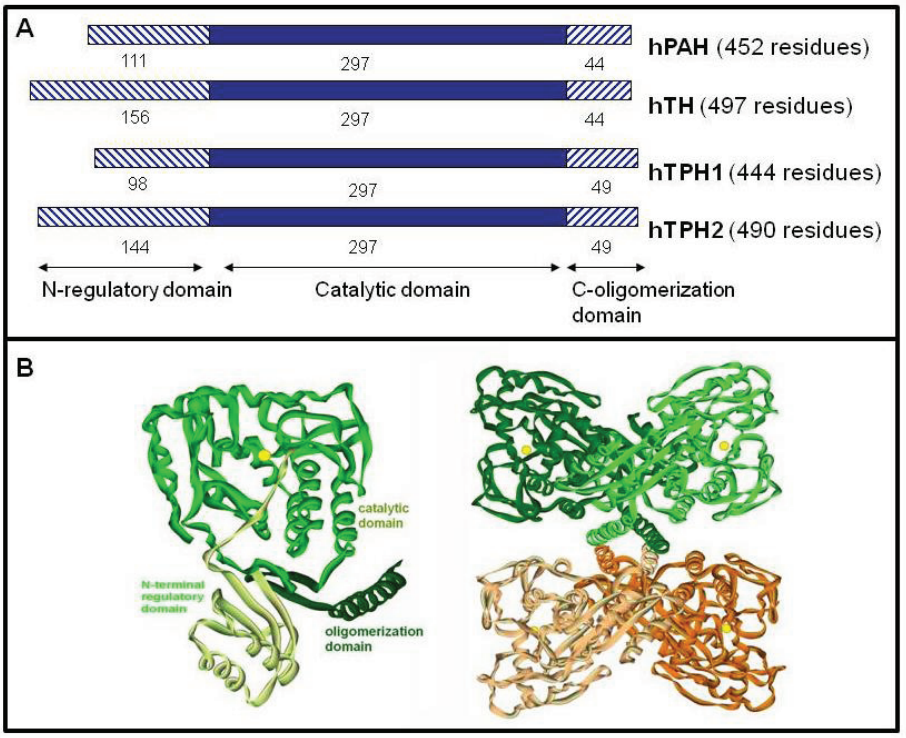


Figure 4. Structure and domain organization of the human AAAH. A) Schematic drawing of the three different domains present in the human enzymes. B) The structural model of monomeric (left) and tetrameric (right) PAH. Composite model created using the structures PDB 2PHM (rat) and PDB 2PAH (human).

1.1.4.1. PAH

The N-terminal regulatory domain of the AAAH is classified as an ACT domain (Siltberg-Liberles and Martinez, 2008) and shows little sequence similarity. In PAH, there is a 30-residue sequence stretch located N-terminal to the ACT domain, referred to as IARS (intrinsic auto regulatory sequence) (Kobe et al., 1999). The IARS includes the phosphorylation site, and has been related to the allosteric regulation of the enzyme by L-Phe (Kobe et al., 1999; Liberles et al., 2005).

Mammalian PAH has been demonstrated to be in equilibrium between dimers and tetramers, with the tetramer being a high activity form of the enzyme whereas the dimer is a low activity form (Martínez et al., 1995; Knappskog et al., 1996). Increase of L-Phe concentration shifts the equilibrium towards the tetramer, what correlates with

PAH activation (Døskeland et al., 1982; Martínez et al., 1995). The tetramer is not completely symmetrical, and it can be considered a dimer of two dimers of different topology (Fusetti et al., 1998).

The human *PAH* (*hPAH*) gene is located in chromosome 12q23.2 (Scriver, 2007). The *hPAH* genomic sequence and its flanking regions contain around 171.000 base pairs although the codifying region, with 13 exons, represents only 3% of the total sequence (Scriver et al., 2003). The *hPAH* gene codifies for only one enzymatic isoform.

1.1.4.2. TH

The N-terminal domain of TH is quite complex, with 4, or even 5 depending on the authors, phosphorylation sites (Dunkley et al., 2004). The multiple phosphorylations have been related to the tight regulation of the enzyme, controlling either kinetic activity or/and binding to protein partners. We will further discuss TH phosphorylation elsewhere (section 1.1.5.2). The boundary between the regulatory and the catalytic domain is located around the residues 165-179 (Goodwill et al., 1997), and the last 50 residues in the C-terminal constitute the oligomerization domain.

TH is tetrameric, and dimeric forms of the enzyme have not been detected *in vivo*. Furthermore, TH is a symmetric tetramer with four identical subunits, in comparison with the asymmetric PAH tetramer (Fusetti et al., 1998).

The human *TH* gene (*hTH*) (Nagatsu, 1989) is located in chromosome 11p15.5, spanning around 8 kilobases and containing 14 codifying exons. It codifies for four different isoforms, created by alternative splicing (hTH1-4), although latter studies have revealed that more mRNA species may be expressed in the cell (Dumas et al., 1996; Ohye et al., 2001; Roma et al., 2007). The hTH1-4 isoforms are expressed in the same tissues, although in different proportions, and share similar kinetic properties, being hTH1 the more active and abundant form (Kaufman, 1995). Nowadays, the physiological significance of the TH isoforms is not completely understood, being an interesting field for future experiments (Kaufman, 1995; Nagatsu, 1995).

1.1.5. Short term regulation of PAH and TH

As it corresponds to enzymes involved in important metabolic processes, the AAAH are well regulated proteins. There are two classes of regulation, short term (seconds-minutes scale) and long term (hours-days scale) regulation. Short term regulation works at the protein level and it is a fast process in response to acute stimuli. On the other hand, long term regulation affects gene expression and it is usually a slower process reflecting continuous stimulus (Stachowiak et al., 1990; Patel and Korotchkina, 2006). In the following paragraphs we will focus on some aspects of the short term regulation of PAH and TH. The major divergence at the N-terminal domain level makes reasonable to assume that the highest differences will be found for the regulatory mechanisms.

1.1.5.1. PAH is principally regulated by phosphorylation and the concentrations of substrate and BH_4

L-Phe is an allosteric regulator of PAH enzymatic activity (for definition and discussion of allosterism, see (Monod et al., 1963) and (Changeux and Edelstein, 2005) for a more recent discussion). The L-Phe substrate binds to the protein inducing a conformational change that produces two related effects (Kaufman, 1993). First, PAH preincubated with L-Phe behaves as an activated enzyme with higher specific activity compared with the non incubated form. This phenomenon seems to be related to movements of the N-terminal domain, increasing the accessibility of the catalytic site, since deletion mutants of PAH lacking the regulatory domain show increase affinity for L-Phe and do not need this preincubation to show maximal activity (Stokka and Flatmark, 2003; Thórólfsson et al., 2003). Secondly, L-Phe binds to PAH with positive cooperativity (for some reviews about cooperativity mechanisms of enzymes, see (Perutz, 1989; Koshland and Hamadani, 2002; Laskowski et al., 2009)), with a Hill coefficient (h) of 2, which represents the conformational change of PAH accompanied by an increase in substrate affinity and catalytic efficiency (Phillips et al., 1984; Thórólfsson et al., 2003). The molecular basis for the activation and positive cooperativity in PAH is not completely understood since there is no available structure of full-length PAH in the substrate-bound form. Nevertheless, limited proteolysis and site directed mutagenesis studies with deletion mutants have revealed important

information about the allosteric mechanisms (Abita et al., 1984; Knappskog et al., 1996).

Another mechanism of short term regulation, widely spread among metabolic enzymes, is phosphorylation. Mammalian PAH is phosphorylated at Ser16 by cAMP dependent protein kinase A (PKA). Upon phosphorylation, PAH shows an increase in basal activity and higher affinity for the substrate, which translates into an activation of the enzyme (Phillips and Kaufman, 1984; Miranda et al., 2002).

Finally, an additional regulatory mechanism is provided by the cofactor. BH_4, besides working as the cofactor of the reaction, also induces a conformational change in PAH structure that results in inhibition and stabilization of the enzyme (Mitnaul and Shiman, 1995; Teigen and Martínez, 2003; Pey et al., 2004; Pey et al., 2004).

1.1.5.2. TH is principally regulated by the catecholamine products and phosphorylation

In contrast to PAH, the effect of the substrate on TH activity and the possible regulatory consequences of this effect *in vivo* are not completely understood. High concentrations of L-Tyr (>50 μM, which is similar to the concentration of this amino acid in the bloodstream and the interior of cells) seem to inhibit specific activity, but there is no physiological explanation for this phenomenon at the moment. Experiments with deletion mutants demonstrated that substrate inhibition is an allosteric mechanism involving the regulatory domain (Quinsey et al., 1998).

Another allosteric mechanism that has been described for TH is a negative cooperativity for BH_4 binding (Flatmark et al., 1999), with an *h* value of 0.6-0.5. Negative cooperativity is a rare event in biology and its physiological explanation is not easy (Koshland, 1996). However, Flatmark *et al.* explained this mechanism as a thigh regulation exerted by the cofactor in a very narrow concentration range (Flatmark et al., 1999), corresponding with the low physiological concentrations of BH_4 within some neurons (<5 μM).

One of the most important and well studied regulatory mechanisms, not only in TH but in a vast majority of metabolic enzymes, is the feed-back inhibition by the product, in which the final product of the reaction inhibits the enzymatic activity of the committed step, avoiding excessive accumulation of the metabolite (Okuno and Fujisawa, 1985; Kumer and Vrana, 1996). The final products of the biosynthetic

pathway controlled by TH, the catecholamines, bind to the active site of the enzyme inhibiting the enzymatic activity by quelation of the iron producing an inactive catecholate-ferric complex (Almås et al., 1992), which results in a competitive inhibition *vs* the cofactor (Nakashima et al., 1999). The complex with the iron atom is responsible for the blue-green colour of the inactivated enzyme form (Andersson et al., 1988).

TH also displays regulatory mechanisms by post-translational modifications, being phosphorylation the most extensively studied. The N-terminal domain of hTH contains at least four recognised phosphorylation sites: Thr8 (Ser8 in rat TH), Ser19, Ser31 and Ser40 (Dunkley et al., 2004). The best characterized phosphorylation site is Ser40, which is phosphorylated by PKA and leads to a lower affinity for catecholamines, blocking the inhibitory effect of these biomolecules (Haavik et al., 1990; Kumer and Vrana, 1996). Phosphorylated TH at Ser40 also presents lower K_m for the cofactor (Toska et al., 2002). Other kinases, as calcium and calmodulin stimulated protein kinase (CaMPK) or mitogen activated protein kinase (MAPK), are able to phosphorylate the protein at Ser40, although it is believed that PKA is the principal effector.

TH phosphorylation is a complex process that is not completely understood, either the mechanism or the physiological significance. In fact after more than twenty years of research, the significance of the phosphorylation in other sites than Ser40 is still under extensively investigation. For instance, phosphorylation at Ser31 is only achieved by ERK (extracellular signal-regulated kinase) 1 and 2, and some studies reported an activation of catalytic activity upon phosphorylation (Haycock and Wakade, 1992). Ser8 has only been found to be phosphorylated *in vitro* (Campbell et al., 1986), remaining unclear if it is relevant *in vivo*. CaMPK II has been identified as the kinase for the site Ser19 in several studies (Campbell et al., 1986; Itagaki et al., 1999). Phosphorylation at Ser19 seems to be related to interaction with the proteins 14-3-3, and it is this interaction the responsible for the enzymatic activation of Ser19 phosphorylated TH (Itagaki et al., 1999; Kleppe et al., 2001; Halskau et al., 2009).

1.1.6. Genetic diseases related to PAH and TH

The importance of the AAAH enzyme family is also reflected by their association to human pathologies, including a number of severe rare genetic diseases.

The study of the molecular, catalytic and regulatory properties of these enzymes contributes to the understanding of the molecular mechanism of the associated illnesses.

1.1.6.1. PAH and PKU

Mutations in the *PAH* gene lead to a deficiency in PAH enzymatic activity, with accumulation of the amino acid L-Phe (hyperphenylalaninemia) (Waters et al., 1998). This metabolic phenotype is linked to the disease called phenylketonuria (PKU), inherited in an autosomal recessive fashion; in these patients accumulation of the excess of L-Phe in brain leads to mental retardation (Scriver and Kaufman, 2001).

PKU research has had a tradition in Norway since the molecular basis of the disease was discovered by the Norwegian Asbjørn Følling in 1934 (Christ, 2003). Since then more than 500 different mutations in the *PAH* gene have been associated to PKU (the whole list of mutations can be found at the website www.pahdb.mcgill.ca). Some of the mutations produce kinetic defects of the enzyme while others are linked to stability impairment and incorrect folding of the protein (Pey et al., 2007), which allows us to classify PKU as a misfolding disease (Martinez et al., 2008). There is no definitive treatment for PKU (Kim et al., 2004; Scriver, 2007), but several strategies have been developed in order to avoid the mental impairment caused by hyperphenylalaninemia. A low phenylalanine semi-synthetic diet is the first level of manipulation, but it is expensive and difficult to maintain the whole life of the patient (Blau and Scriver, 2004). Supplementation with the cofactor, BH_4, which exerts a protective and chaperone effect on PAH, appears effective depending on the mutation (usually, in mild forms of PKU) (Muntau et al., 2002; Blau and Erlandsen, 2004; Erlandsen et al., 2004). An enzyme substitution strategy with phenylalanine ammonia lyase (PAL) has been developed recently (Sarkissian et al., 1999), and constitutes a promising alternative to special diets.

Epidermal PAH has also been related to a multifactorial disease called vitiligo, characterised by loss of skin colour. The ethiology of vitiligo is largely unknown being the autoimmune hypothesis the most accepted one (Le Poole and Luiten, 2008). Patients with vitiligo have been reported to present an impaired BH_4 synthesis and recycling, with an abnormal accumulation of 7(R,S)-BH_4. These 7-BH_4 cofactor analogues are important inhibitors of PAH, resulting in PAH deficient activity in patients with vitiligo (Schallreuter et al., 2005).

1.1.6.2. TH and autosomal recessive DRD and Parkinson's disease

The scenario with diseases associated to TH dysfunction is far more complicated than for PAH, possibly because of the lethality of mutations in *TH* gene, which has been observed in transgenic mice with this gene disrupted (Zhou et al., 1995). However, specific point mutations (Knappskog et al., 1995; Ludecke et al., 1996; Royo et al., 2005) in the *TH* gene have been linked to the autosomal recessive form of the Segawa's disease, also called Dopa responsive dystonia (DRD) (Ichinose et al., 1999). The autosomal dominant form of the disease was first described by Segawa (Segawa et al., 1976) as a dystonia with onset in the childhood and responsive without side effects to L-DOPA, and it is produced by mutations in the *GTPCHI* gene (Nygaard et al., 1993; Nagatsu and Ichinose, 1996).

Parkinson's disease is characterised by degeneration of dopaminergic neurons in the *substantia nigra,* affecting the motor capacities of the patient. The causes and mechanisms of this pathology are not completely clear, but it seems that TH plays a role in the mechanism of the disease, since dopaminergic neurons, where TH is abundantly expressed, are the first ones to degenerate (Haavik and Toska, 1998). For reviews about TH and dopamine related diseases, see (Nagatsu and Ichinose, 1999; Haavik et al., 2008).

1.1.6.3. BH_4 and human genetic diseases

Diseases related to BH_4 deficiency were first described associated to a type of PKU (atypical or malignant PKU) unresponsive to dietary treatment (Scriver et al., 1995). Later it was seen that the hyperphenylalaninemia was accompanied by low levels of neurotransmitters (dopamine and serotonin) in cerebrospinal fluid (CSF). These diseases constitute a heterogeneous group where we can find mutations in different genes, both in the synthesis and recycling pathways (Blau et al., 2001).

As we have mentioned previously, mutations in the *GTPCHI* gene generate the dominant form of DRD (see paragraph *1.1.6.2*) or Sewaga's disease (Thony and Blau, 2006). PTPS deficiency is the most frequent form of BH_4-related diseases, while PCD and DHPR, the recycling enzymes of BH_4, are also implicated in human diseases, being DHPR deficiency the most severe one, while PCD deficiency is presented with

hyperphenylalaninemia and persistent high urinary levels of primapterin (Thony et al., 1998). For a review on BH_4 related diseases see (Longo, 2009).

1.1.7. Tryptophan hydroxylase (TPH)

Although this work mainly focuses on PAH and TH, TPH activity has also been considered. TPH is the rate limiting enzyme in the synthesis of serotonin or 5-hydroxytriptamin (5-HT), hydroxylating tryptophan into 5-hydroxytryptophan. It is also implicated in the synthesis of melatonin, a derivative of serotonin. The hydroxylation mechanism is similar to that of TH and PAH, requiring for the catalysis BH_4, ferrous iron and oxygen.

Probably the most important difference between TPH and TH and PAH is the fact that in mammals two independent genes codify for two different TPH isoforms, TPH1 and TPH2. Although the site of expression of these two genes is controversial in the literature, they are referred to as peripheral *TPH1* (predominantly expressed in pineal gland, mast cells and enteric neurons) and central or neuronal *TPH2* (expressed in mesencephalic tegmentum, striatum, and hippocampus in the central nervous system) genes, reflecting the main tissues where they are expressed (Sakowski et al., 2006; Haavik et al., 2008).

Serotonin and melatonin function as neurotransmitters and hormones, controlling important behaviours, as appetite, sleep, memory, body temperature, mood or sexual behaviour, among others (Lucki, 1998). Moreover, some polymorphisms in the *TPH* genes have been associated with mood disorders, as depression or schizophrenia (Cichon et al., 2008; McKinney et al., 2009).

1.2. The nematode *Caenorhabditis elegans*

1.2.1. C. elegans as a model system in modern molecular biology: an introductory overview

*C. eleg*ans is a free living nematode worm that lives in the soil (see Figure 5 for an overview of the worm morphology), and became famous 45 years ago, when Sydney Brenner proposed the worm as a model system to study development and neurobiology, between others (Brenner, 2009). Since then, the number of laboratories using *C. elegans* has increased in an exponential manner (Hoffenberg, 2003). But what are the reasons for the success of such a small animal that has already received three Nobel prizes? (About these important *C. elegans* achievements see (Barbour, 2002; Zamore, 2006; Zimmer, 2009)).

C. elegans is considered a model organism in biology, presenting important advantages among other organisms, as already seen and exposed by Brenner in his first article on the nematode (Brenner, 1974) and we here proceed to enumerate a few of them (for reviews on the use of *C. elegans* as a model organism see (Artal-Sanz et al., 2006; Kaletta and Hengartner, 2006)):

- A fast development. At 20 ^{o}C completed life cycle is achieved in 3-4 days.
- There are two sexes. Although the main gender is hermaphrodite, 0.1% of the population are males. This rare feature is essential to maintain and isolate genetic strains, but the existence of males allows crossing strains carrying different mutations.
- Production of large numbers of offspring. Around 300 eggs are laid by the hermaphrodite by self-fertilization, and around 1000 by male-mating.
- Easy and inexpensive laboratory conditions. The worms are maintained on Petri dishes on agar, seeded with a bacterial lawn as food source.
- Relatively simple anatomy. Approximately 1000 cells constitute the whole body, (959 somatic nuclei for the hermaphrodite and 1031 for the male). Despite this simplicity, complicated structures and behaviours characterize this organism.
- *C. elegans* was the first multicellular organism whose genome was sequenced, which consists of just over 100 million base pairs and 20,000 genes (The *C. elegans* Sequencing Consortium, www.wormbase.org).

- Survival to freezing mechanisms. Worms stocks can be stored frozen in liquid nitrogen, being viable during decades.
- Transparency. An important feature, since it allows us to distinguish anatomical structures and to follow fluorescence markers *in vivo*.

Since Brenner´s initial studies, investigation with *C. elegans* has not stopped, with new techniques and methods developed every year. 4D microscopy, green fluorescence protein (GFP) tagging and visualization *in vivo*, silencing at specific stages or in specific groups of cells, electrophysiology of single neurons and other experimental procedures are nowadays routine work in *C. elegans* laboratories. Research fields are extensive, but important advances have been developed regarding interesting topics as ageing, programmed cell death, obesity or plasticity of the nervous system (for reviews in some of these topics see (Hobert, 2003; Putcha and Johnson, 2004; Olsen et al., 2006)).

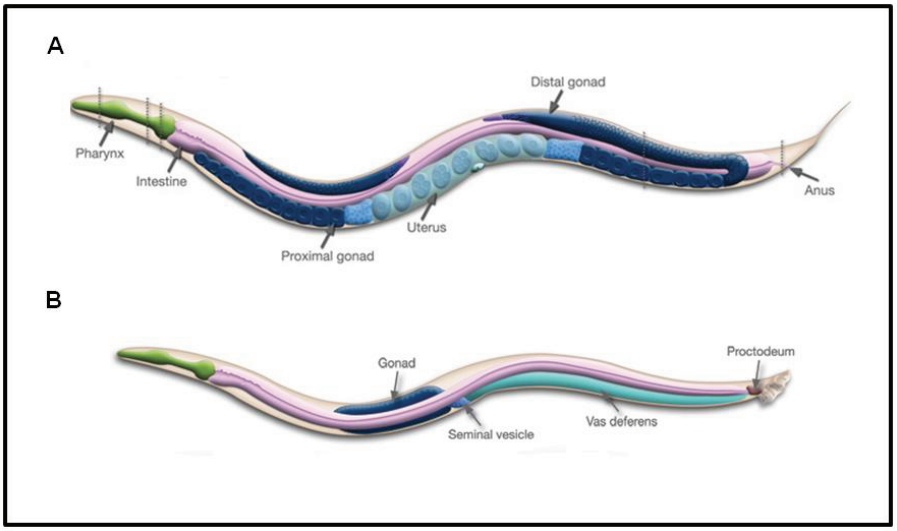


Figure 5. *C. elegans* general body plan. A) Hermaphrodite anatomy. B) Male anatomy, with focus on differential structures. *Figure adapted from* www.wormatlas.org

1.2.2. Evolutionary relationships

C. elegans belongs to the phylum of nematoda, in which we can find free-living nematodes (as *C. elegans* and *C. rabditis*) and parasitic nematodes (*Ascaris sum* and

Brugia malayi, between others). Nematodes are located in the evolution tree in a deep branch close to the separation between arthropod and vertebrate lineages. Although they are far from mammals, it has been shown that almost all lower and higher animals share a great variety of biological processes and molecules (Blaxter, 1998; Cutter et al., 2009). Biological processes are well conserved across animal kingdom, and the main difference seems to be the degree of complexity of these processes. As an example to understand this idea, around 60-80% of the genes in *C. elegans* genome have the corresponding version in the human DNA (Harris et al., 2004).

It has been seen that protozoans, as *Dyctiostelium discoideum* or *Leishmania*, only present one copy of the AAAH in its genome, whereas metazoans, such as *C. elegans* or *D. melanogaster*, own at least three different genes of the AAAH family. Characterization of PAH from the mould *D. discoideum* (Siltberg-Liberles et al., 2008) confirmed that PAH was the ancestor of the AAAH. This ancestral gene from protozoans must have been duplicated twice, in agreement with the three copies of the hydroxylases found in the *C. elegans* genome, generating the new functions of TH and TPH (Figure 6).

1.2.3. C. elegans as a model organism for human diseases

Once that we have explained the advantages of *C. elegans* and the evolutionary relationship with other species, it is reasonable to select the nematode to study important human diseases. Let's consider the two possible scenarios that we can find (Culetto and Sattelle, 2000; Kaletta and Hengartner, 2006):

- If the gene of interest has an ortholog in the *C. elegans* genome: apart of expression pattern experiments to see localization or cDNA cloning to study protein properties *in vitro* (between others), knocking out or knocking down the gene of interest is an available tool to study gene function. As an example, the human gene responsible for Duchenne muscular distrophy has its counterpart in the worm genome, and loss-of-function animals exhibit muscular contractions defects (Bessou et al., 1998). Here, *C. elegans* emerges as a good model to get further knowledge on this disease. In this thesis several worms knocked-out in genes related to AAAH and their human genetic diseases have been studied and characterised.

- If the gene of interest has no ortholog in the *C. elegans* genome: even then there is a possibility to model the human disease. Microinjection of the corresponding human cDNA into the gonads of the animal will produce a transgenic offspring expressing the human protein under a specific promoter. Transgenic models of Alzheimer's disease and Parkinson's disease have been developed in *C. elegans* with this technique and are the choice model to study neurodegenerative diseases in many cases (Link, 1995; Link, 2005; Teschendorf and Link, 2009). This strategy has not been pursued in the present work, although the possibility to inject disease-related mutant constructs of the human PAH, TH and TPHs to study its *in vivo* behaviour is extremely interesting.

1.2.4. The AAAH family in C. elegans

The *C. elegans* genome is predicted to have three different genes encoding three AAAH (Figure 6). The gene K08F8.4 in *C. elegans* genome was predicted to encode a putative PAH, while the genes B0432.5 and ZK1290.2 were proposed to encode putative TH and TPH, respectively.

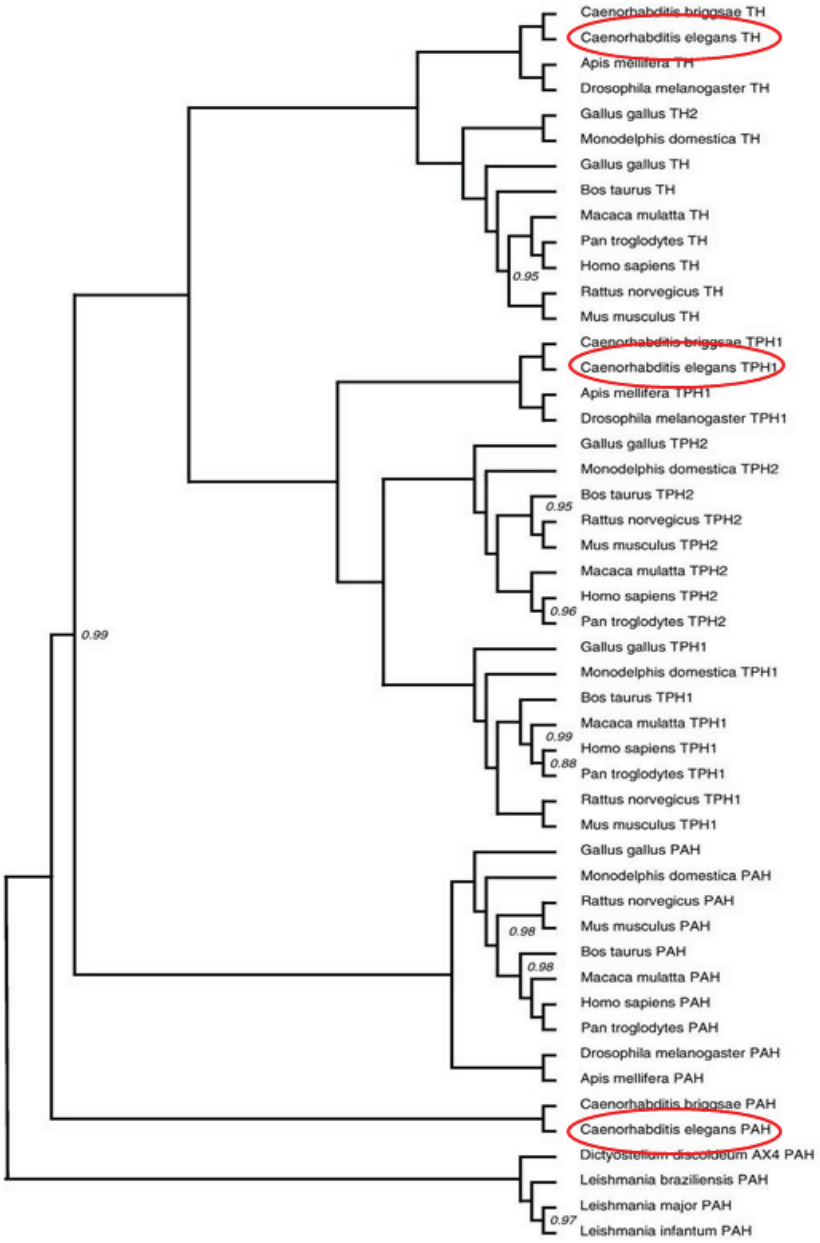


Figure 6. Phylogenetic tree of the AAAH, rooted with PAH from the protozoa as an outgroup. The three AAAH from *C. elegans* are circled in red. *The figure has been adapted from (Siltberg-Liberles et al., 2008)*

1.2.4.1. PAH is expressed in hypodermis

Since PAH is expressed in hypodermis and in close relation with the epithelial system, a brief description of these organs will be presented here. The hypodermis is composed by a main body syncytium (hyp-7), some extra hypodermal cells in the head and the tail (hyp1-hyp11), and the seam cells (Figure 7A), a kind of blast cells responsible for alae production in the cuticle. The principal functions of the hypodermis are nutrient storage, secretion of the adjacent cuticle and engulfment of apoptotic cell bodies.

The cuticle is a flexible and resistant exoesqueleton that completely surrounds the body of the nematode. It is implicated in body morphology, correct locomotion and protection from the environment. It is synthesised five times, once in the embryo and another four at the end of each larval stage. The cuticle presents four different layers, from the outer to the inner part: epicuticle, cortical, medial and basal cuticle (Figure 7, B). The molecular components of this exoesqueleton are collagens, non-reducible cuticlins and a surface coat of lipids and glycoproteins. Both collagens and cuticlins are highly cross-linked through di, tri and iso tyrosine bridges, which makes the cuticle a very resistant structure (www.wormbook.org).

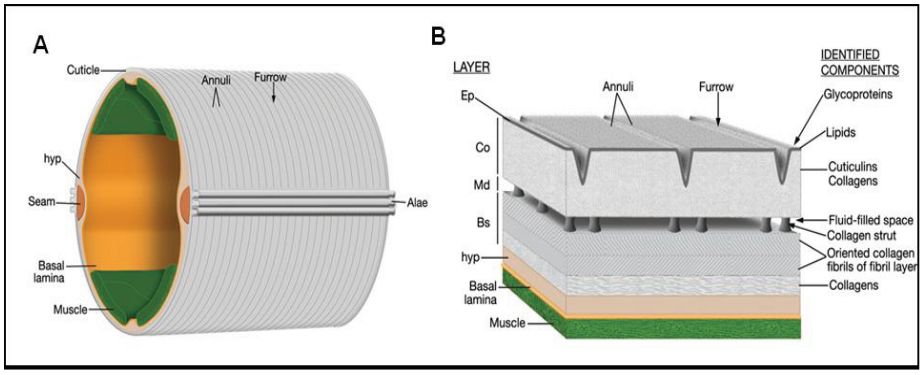


Figure 7. The cuticle of *C. elegans*. Representative pictures of the components of cuticle and hypodermis in longitudinal (A) and transversal (B) sections. *The figures have been modified from its originals in www.wormatlas.org.*

Loer *et al.* cloned and sequenced the nematode *PAH* gene (K08F4.8) (Loer et al., 1999). Expression pattern, seen by LacZ (β-galactosidase) reporter fusion constructs and confirmed by immunostaining with mammalian PAH antibody PH8, revealed that

PAH is expressed mainly in the cytoplasm of hypodermal cells, with a strong anterior-posterior gradient (Loer et al., 1999). Bacterial expression of K08F4.8 cDNA was performed and PAH activity was measured in crude extracts, confirming the PAH function of the gene and also low, but detectable, TPH activity (no TH activity was detected) (Loer et al., 1999).

In spite of these experiments confirming the presence of a gene encoding PAH in *C. elegans* genome, at the start of this thesis project no studies had been reported on PAH function in nematodes. Loer *et al.* proposed a role of PAH involved in synthesis and maintenance of cuticle structures (Loer et al., 1999). A supply of tyrosine would be provided by PAH and used for tyrosine cross-linking of collagens and cuticlins (Fujimoto et al., 1981; Yang and Kramer, 1999).

1.2.4.2. TH and TPH are expressed in the nervous system

More studies are available in relation to TH and TPH function in the nematode, due to its importance in the synthesis of neurotransmitters, dopamine and serotonin. A brief introduction of both these enzyme systems will be given here.

TPH and serotonin

The gene ZK1290.2 (*tph-1*) is expressed in serotonergic neurons and is involved in several processes, as metabolic control of food ingestion and reproduction and egg-laying rate, between others (Nuttley et al., 2002; Estevez et al., 2004). Mutants bearing a deletion on the *TPH* gene (*tph-1* mutants) are defective in serotonin synthesis (Sze et al., 2000).

TH and dopamine

The gene B0432.5 (*cat-2*) was identified as a putative TH (Lints and Emmons, 1999), and mutants lacking this gene (*cat-2* mutants) were defective in dopamine synthesis. Experiments carried out with GFP reporter fused to the *cat-2* gene confirmed that TH was expressed in the catecholaminergic system.

There are 8 dopaminergic neurons in *C. elegans* hermaphrodite (Figure 8) (Sulston et al., 1975): four symmetrically cephalic cells (CEPs), two bilateral anterior deirids (ADEs) in the head and two bilateral posterior deirids (PDEs) in the middle part of the body. Moreover, males contain three extra pairs of dopaminergic neurons in the

tail (Sulston et al., 1975; Lints and Emmons, 1999). Already from the first observations these neurons were considered to be mechanosensory, since their ciliated endings of the dendrites are embedded in the sub-cuticle in contact with the exterior world, a perfect position to sense diverse stimuli from the environment (for a good review on dopamine in the worm, see (Nass and Blakely, 2003)).

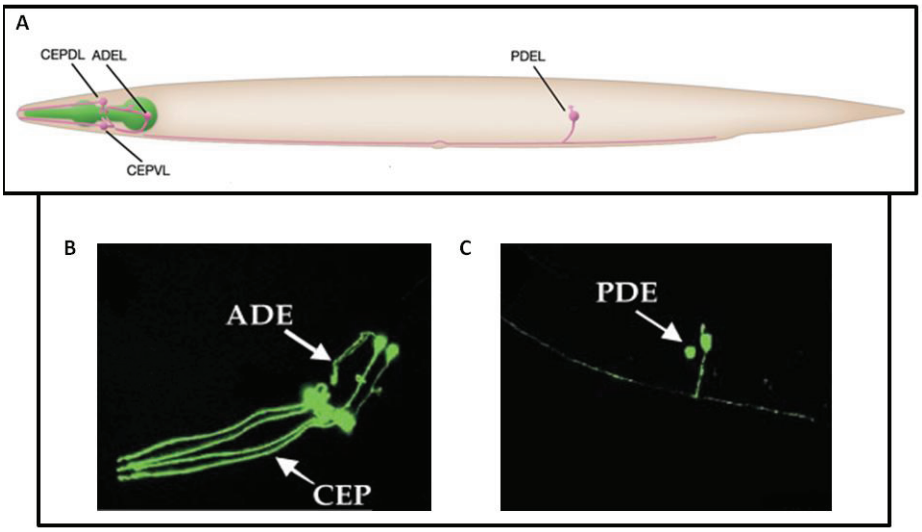


Figure 8. Dopaminergic neurons in *C. elegans*. A) Schematic cartoon showing the left side of neurons expressing dopamine (CEPD, CEPV, ADE and PDE) in the hermaphrodite. B and C) Green fluorescence protein (GFP) expressed under dat-1 (dopamine transporter) promoter is only seen in dopaminergic neurons in the head (B) and the medial part (C). *A) has been taken from www.wormatlas.org, and B) and C) from (Nass and Blakely, 2003).*

The possibility of a mechanosensory function of dopamine was confirmed by Sawin *et al*. (Sawin et al., 2000) in their experiments with *cat-2* knock-out mutants, demonstrating that dopamine controls the context-dependent locomotion in response to food. Dopamine is involved in what it is called "Basal slowing response", consisting on the slower rate of movement that well-fed animals present in the presence of a bacterial lawn than in the absence of the food source. *Cat-2* animals do not have this behaviour. But the availability of food also regulates locomotion with, at least, one extra mechanism, which is called the "Enhanced slowing response". This is exhibited by starved animals when they encounter a bacterial source, moving much more slowly than if they are not starved. Contrary to the basal slowing response, this behaviour is

mediated via serotonin. In Nature, both mechanisms are supposed to be very important for survival, since feeding is a basic and primary necessity for *C. elegans*. Dopamine has also been implicated in the process of learning and behavioural adaptation (Sanyal et al., 2004; Kindt et al., 2007; Lee et al., 2009), and in the male-mating behaviour (Loer and Kenyon, 1993), what is expected since the extra dopaminergic neurons of the males are associated with the copulatory apparatus.

1.2.4.3. TH and dopamine from other organisms

Extensive research has been done with catecholamines in different organisms. *D. melanogaster* is another model organism used for biological research (Burdett and van den Heuvel, 2004; Ashburner and Bergman, 2005), and we have extensive information about TH protein from the fly. There is only one gene for TH in *D. melanogaster*, although two different isoforms of the protein are generated by alternative splicing (Neckameyer and Quinn, 1989; Birman et al., 1994). One of them is expressed in nervous system and its physiological role is the synthesis of catecholaminergic neurotransmitters; on the contrary, the second one is expressed in hypodermis and it has been described as an enzyme implicated in the pigmentation and hardening of the cuticle. Moreover, animals lacking the *th* gene are characterized by unpigmented embryos unable to hatch (for further information on the TH function in the hypodermis of *Drosophila* see (Davis et al., 2007)). These two isoforms present important differences in regulation of the activity, especially concerning two important mechanisms of TH regulation in mammals, i.e. feed-back inhibition and phosphorylation (Vie et al., 1999). The hypodermal isoform appears comparatively less sensitive to dopamine inhibition than the neuronal isoform, while the later is activated through phosphorylation by PKA also in the absence of dopamine.

Different invertebrates have been used to study TH activity and catecholamine biosynthesis. The presence of dopamine in the central nervous system of crustaceans has been demonstrated and it appears to be related, between other roles, to adaptative behaviour (Tierney et al., 2003). In the cuttlefish *Sepia officinalis*, TH is expressed in the ink defence system. *S. officinalis* appears to use melanin as a primitive immune mechanism and dopamine is implicated in melanin synthesis and regulation (Fiore et al., 2004).

1.3. Natural and chemical chaperones

As already mentioned (see section 1.1.6.1), most PKU mutations are linked to impaired folding and stability of the protein (Pey et al., 2007), and PKU is one of the metabolic misfolding diseases most studied to date (Martinez et al., 2008). There are not as many different mutations in the *TH* gene as in the *PAH* gene, but some of them have also been reported to exert decrease thermal stability (Royo et al., 2005). Based on the identification of disease-associated mutants of the AAAH as rather unstable proteins, new therapeutic strategies aiming to increase protein stability are arising for these diseases.

1.3.1. The quality control system of the cell

Folding inside the cell is an extremely important and regulated process that ensures correct protein stability and function. The quality control system (QCS) in the cell monitors that proteins are correctly folded, assists them if they are not able to fold by themselves, and targets the unfolded proteins for degradation. The QCS has two principal members: the chaperones and the proteasome. Here we proceed to introduce them briefly; for detailed information see (Trombetta and Parodi, 2003; Bukau et al., 2006) (Figure 9).

Molecular chaperones and cochaperones are a set of proteins that bind to partially unfolded peptides, usually via hydrophobic exposed regions, and help these peptides to fold correctly (Liberek et al., 2008). Probably, the most extensively studied chaperone is the bacterial GroEL and its cochaperone GroES, which share several properties with the mammalian chaperones (Fenton and Horwich, 1997; Sigler et al., 1998).

The ubiquitin proteasome pathway (UPP) is the main route for protein degradation. If a protein is unfolded or incorrectly folded and it is not able to reach the native conformation even with the chaperone help, it will be targeted for proteasomal degradation. Attachment of at least four molecules of ubiquitin through ordered enzymatic reactions is the key signal for proteasome recognition. The proteasome is a multi domain protein complex that cleaves proteins and peptides into short peptides (for a review on UPP see (Nalepa et al., 2006; Schwartz and Ciechanover, 2009)).

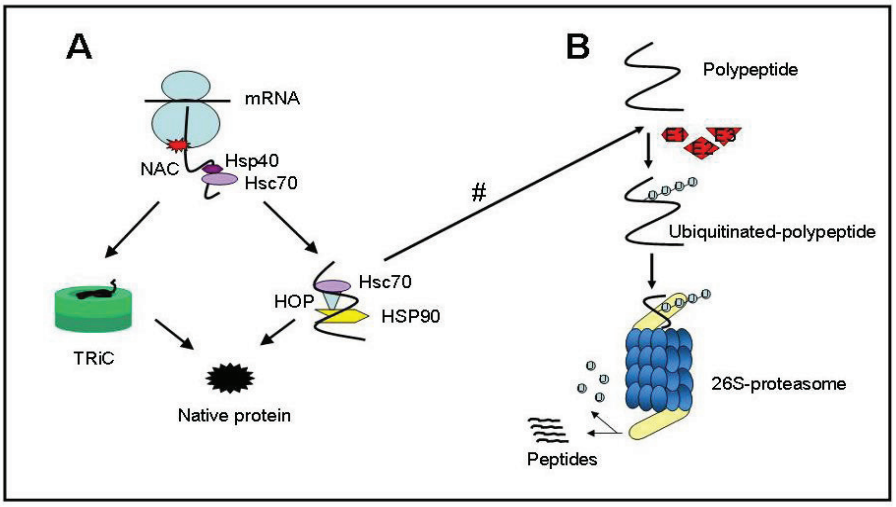


Figure 9. Quality control system (QCS) in the eukaryotic cytosol. A) Protein assisted folding for newly synthesized polypeptides, involving the interactions with different molecular chaperones; NAC, nascent-polypeptide associated complex; HOP, Hsp organizing protein. B) Ubiquitin-proteasome pathway (UPP) for the degradation of defective proteins. U, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase. *The figure has been taken from (Martinez et al., 2008).*

1.3.2. Misfolding human diseases

Customarily, misfolding diseases are classified in two groups (Martinez et al., 2008; Winklhofer et al., 2008):

- *Gain-of-function diseases:* also referred to as amyloid diseases, due to the amorphous deposits of amyloid fibrils found in different tissues and organs in these diseases. The molecular basis of these pathologies is either an overwhelmed QCS or an unfolded protein that is resistant to degradation. In the latter case the unfolded peptides exposing hydrophobic patches are accumulated within the cell, and the protein aggregates into toxic insoluble structures that will finally form the amyloidogenic fibril (Selkoe, 2004; Chiti and Dobson, 2006; Luheshi and Dobson, 2009).
- *Loss-of-function diseases*: Though most proteins will form fibers at selected *in vitro* conditions, only a limited number of proteins actually form amyloids *in vivo* (Lopez de la Paz and Serrano, 2004). Thus, most misfolded proteins are completely degraded by the proteasome with no intracellular accumulation of aggregates, and as a result no specific protein function is retained. In fact, most

of the genetic misfolding diseases belong to this group, including metabolic and endoplasmic reticulum (ER) trafficking-diseases, such as cystic fibrosis, one of the misfolding illnesses most studied in the literature (Amaral, 2004; Amaral, 2006). In this group we also find PKU (Pey et al., 2007).

1.3.3. Natural and pharmacological chaperones

An important therapeutic aim in relation with misfolding diseases is to promote the correct folding and restore protein activity (pharmacological rescue). One of the strategies to recover proper folding is by using small molecules called chaperones. These molecules will bind to native or partially folded states of the protein and will promote folding and stabilization (Kolter and Wendeler, 2003).

1.3.3.1. Chemical chaperones

Chemical chaperones are small organic molecules, such as osmolytes (glycerol, sucrose…), dimethyl sulfoxide (DMSO), trimethylamine or even detergents and phospholipids, that have been proved to stabilize protein structure (Tatzelt et al., 1996). Despite their initial promising potential, soon it was obvious that they presented some important disadvantages. Since they are not specific stabilisers of one protein (unspecific binding and stabilization) high amounts are needed to see a biological effect, and these high doses are usually toxic for the cell, as found in the case of stabilization of mutants associated with cystic fibrosis (Sato et al., 1996; Wang et al., 2007).

1.3.3.2. Pharmacological chaperones

On the other hand, pharmacological chaperones are low molecular weight compounds specific for one protein or family of proteins that have a stabilizing effect at low concentrations (Ulloa-Aguirre et al., 2004). One of the disadvantages of these chaperone molecules is that they usually bind very tightly and/or to functional sites within the protein, functioning as inhibitors. A compromise between binding and inhibition must be found.

A recent strategy to find specific pharmacological chaperones is the use of high throughput (HTP) screening with chemical libraries, containing a broad set of

representative organic compounds. The HTP methodology allows the researcher to search a determined set of positive hits within a library of thousands of putative compounds (Tropak and Mahuran, 2007). The number and type of available commercial libraries increases steadily, each one adapted to specific research needs and applications.

Some examples about protein rescue through pharmacological chaperones in relation to misfolding diseases can be found in the following articles: (Parenti, 2009) in lysosomal storage diseases, (Yam et al., 2006) in Fabry's disease or (Amaral, 2006) in cystic fibrosis.

1.3.3.3. Natural chaperones

Natural chaperones are specific cases of pharmacological chaperones, since they are natural substrates, cofactors or inhibitors of the studied protein and their *in vivo* concentration can be increased therapeutically. These natural ligands have stabilising effects, apart from their biological role in the catalysis or physiological function of the protein (Martinez et al., 2008). Therapeutic supplementation with these biomolecules arises as a possibility to treat misfolding diseases, as it has been seen with the role of vitamins stabilising some proteins (Li et al., 1998; Ames et al., 2002). Also in the case of treatment with natural chaperone compounds dose-dependent toxicity should be taken into account.

1.3.4. Therapeutic application of natural and pharmacological chaperones in the treatment of PKU

As most PKU mutations are related to misfolding and decreased stability of the native state of the protein (Gjetting et al., 2001; Pey et al., 2003; Pey et al., 2007; Scriver, 2007), the use of natural and pharmacological chaperones arises as a good candidate for clinical treatment. Actually, supplementation with BH_4 is already established as a PKU therapy in some patients with mild forms of the illness (see paragraph 1.1.6.1) (Erlandsen et al., 2004; Levy et al., 2007). BH_4 seems to exert its therapeutic role through different mechanisms, including a natural chaperone effect in PAH, stabilizing the structure of the enzyme (Pey et al., 2004; Thony et al., 2004; Scavelli et al., 2005).

Pey *et al*. have recently demonstrated the usefulness of HTP screening of chemical libraries to find putative pharmacological chaperones to treat PKU (Pey et al., 2008). The authors identified a set of compounds that stabilised recombinant PAH. Moreover, experiments with disease-related mutants in cellular animal cultures as well as with wild type mice supplemented with these organic molecules further established their potential as putative therapeutic agents.

2. Aims of the study

The main goal of this thesis was to comparatively study the AAAH from humans and nematodes, in order to unravel evolutionary and novel functional aspects of this enzyme family. A second goal of this work was to analyze the effect of small molecules as stabilizers of the conformation of TH, PAH and TPH. These molecules have a potential as therapeutics for diseases related to AAAH, based on the chaperone concept. In order to achieve these goals, we have focused on two main subprojects, with the following partial aims:

2.1. The family of AAAH in *C. elegans* (Papers I-II)

- PAH is a well studied protein in mammals, possible due to its implication in the disease PKU. On the other hand, little is known about the function and regulation of this enzyme in invertebrate organisms. We aimed here to get deeper insights into the cePAH characteristics and functions, both *in vitro* and *in vivo*, and to extract evolutionary information on PAH. Moreover, this information is expected to provide a better understanding of the function and regulation of the human enzyme both in health and disease.
- We also aimed to investigate another important AAAH in *C. elegans*, i.e. TH, which required the cloning and characterization of the recombinant enzyme (ceTH). In addition of being important for the understanding of adaptive changes along evolution in this enzyme family, this study is expected to provide the basis for the investigation of the physiological relevance of important regulatory mechanisms established for mammalian TH, such as feed-back inhibition by catecholamines or N-terminal phosphorylation, using a simpler and easy to handle organism, like *C. elegans*.

2.2. Stabilization of the AAAH through chaperone molecules (Papers III-IV)

- BH_4 is considered a chaperone of PAH conformation, and it is used for the treatment of PKU patients, notably those with mild forms of the disease. This

BH_4 supplementation therapy is supposed to increase both PAH activity and stability. Moreover, BH_4 is also used in the therapy of other diseases, as hypercholesterolemia or diabetes mellitus. We attempted in paper III to unravel if BH_4 is able to cross the BBB at therapeutic doses and if so, to study the effect upon TH. We also aimed to investigate a potential use of BH_4 as chaperone for the treatment of DRD associated to the misfolding of TH mutants.

- HTP screening of PAH detected four compounds with potential as pharmacological chaperones that increased PAH (wild-type and mutant) stability. In paper IV, we aimed to comparatively study the effect of these four compounds on PAH, TH and TPH2 stability. The close relationship, regarding both structure and catalytic activity, of the members in this enzymatic family points to the importance of comparative studies, especially when the goal is to develop new and specific therapies.

3. Results and contributions

3.1. The AAAH in *C. elegans* (PAH and TH)

3.1.1. C. elegans PAH (cePAH) is implicated in the synthesis of a melanin-like compound (Paper I)

Previous studies by Loer *et al.* focused on the cloning and expression of the recombinant PAH from the nematode *C. elegans* and confirmed that the K08F8.4 gene encoded a PAH (Loer et al., 1999). Kinetic characterization of recombinant cePAH revealed similar catalytic properties in comparison with human PAH (hPAH): high specific activity and similar apparent affinities (K_m) for the substrate, cofactor and O_2, and similar substrate and cofactor specificity. On the other hand, the regulatory behaviour presented several differences. cePAH does not need to be pre-incubated with the substrate, L-Phe, to reach maximal activity. It is also devoid of the positive cooperativity induced by Phe, a regulatory mechanism of mammalian PAH that seems relevant for the need of avoiding the toxic accumulation of this amino acid. A detailed study of the *pah-1* mutant, lacking cePAH in the worm, was performed in order to elucidate the *in vivo* function of the enzyme. The *pah-1* worms seemed apparently healthy with no obvious phenotype. Since cePAH is expressed in hypodermal cells (Loer et al., 1999) special focus was given to the cuticle. The structure of *pah-1* mutant cuticles, in contrast with *cat-4* mutants, which are defective in GTPCH, appears to be normal, as studied by different microscopy approaches. Mechanical resistance was studied by sonication but no significant differences were observed between wt (N2) and *pah-1* animals. In contrast, the *bli-3* strain, which is defective in the enzyme Doux (dual oxidase) responsible for di-tyrosine cuticle cross-linking and that presents cuticle defects such as blisters and disattachment of the cuticle to the hypodermis, is highly sensitive to sonication. Interestingly, treatment with hydrogen peroxide revealed dramatic differences between *pah-1* and wt strains, the *pah-1* mutants being much more resistant to the oxidizing conditions. Isolation and characterization of wt and *pah-1* (and *cat-4*) cuticles subjected to acid hydrolysis revealed a compound almost absent in *pah-1* and *cat-4* mutants but not in wt. Further purification and physico-chemical characterization of this molecule identified it as a putative melanin-like compound of

the family of pheomelanins. The fluorescence and infrared spectra of this melanin is incompatible with that of a di-tyrosine. In order to investigate a putative role of this melanin-like compound in anti-oxidant mechanisms, we measured catalase and superoxide dismutase (SOD) activities in wt and *pah-1* extracts. In fact, an important increase in SOD activity, together with a slight increase in worm survival, was detected in *pah-1* mutants. This is most probably due to a compensatory mechanism that further confirms the role of cePAH in the synthesis of a melanin-like compound with anti-oxidant protection function.

3.1.2. Cloning and recombinant expression of C. elegans TH (ceTH) (Paper II)

The gene B0432.5 was predicted to codify a putative TH involved in the synthesis of dopamine (Sawin et al., 2000). In order to get further knowledge on TH function in the worm we aimed to clone and characterise this gene, producing the recombinant protein *in vitro* and studying its function and regulation. Information available in Wormbase (www.wormbase.org) seemed confusing, since the gene structure predicted two spliced isoforms of TH: one of them (B0432.5a) was lacking part of the catalytic domain and the complete oligomerization domain, and the second one (B0432.5b) did not have a complete regulatory ACT domain. Amino acid sequence of these two isoforms and several THs both from invertebrates and vertebrates (including the nematode *C. briggsae*, closely related to *C. elegans*) did not provide coherent sequence alignments. Moreover, every TH protein studied so far presents the typical three domain organization, which is absent in the B0432.5a and B0432.5b isoforms. By using specific primer design we confirmed the existence of a new more reliable isoform (B0432.5c), combination of the a and b isoforms. This c isoform presents the three domains of the AAAH, and it was cloned in this study. The recombinant ceTH protein was expressed in *E. coli* and purified, and its kinetic characterization confirmed its tyrosine hydroxylating activity, and almost no PAH or TPH activity. This is different to the properties of the hTH, which hydroxylates L-Tyr and L-Phe with similar efficiency. Moreover, the specific TH activity was significantly lower than for the human enzyme, but similar or even lower activities have been previously reported for THs from other invertebrate organisms. Special focus was paid to the regulation of the catalytic activity, and in agreement with the divergence of the N-terminal domain, important differences were found between ceTH and hTH. The

apparent affinities for the cofactor were similar, but ceTH does not display negative cooperativity binding for BH_4. The apparent affinity for the substrate is significantly lower for ceTH, and the substrate inhibitory effect at high concentrations of L-Tyr is almost absent. Moreover, the feed-back inhibition by dopamine is much lower in ceTH than in hTH, suggesting that the regulation by the product is not as critical in the worm as in the human. A putative phosphorylation site, homologue to Ser40 in hTH, was found in the sequence of ceTH. As seen by MALDI-TOF mass spectroscopy, Ser35 in ceTH was phosphorylated upon incubation with PKA. PKA phosphorylation does not seem to activate the enzyme, either in the presence or absence of dopamine, as is the case for hTH. Other regulatory effects might be affected by phosphorylation at this site.

3.2. Towards the stabilization of the AAAH: natural and pharmacological chaperones

3.2.1. The cofactor BH_4 functions as a natural chaperone of hTH (Paper III)

The role of BH_4 as a natural chaperone of PAH, stabilising the enzyme conformation upon binding, is well established, both *in vitro* and *in vivo*. On the other hand the effect of the cofactor on TH is confusing, and both destabilization/inactivation of the enzyme by BH_4, with concomitant cell death, and protective effects have been reported. Moreover, the final catecholamine products are also known to have a stabilising role in TH conformation. In this work we thus set up to get insights on the effect of BH_4 and dopamine on TH stability and activity, both *in vitro* and *in vivo*.

The circular dichroism (CD) unfolding kinetics of recombinant hTH1, in absence or presence of BH_4, revealed a concentration dependent stabilization exerted by the cofactor. Same experiments with other synthetic BH_4 analogues (BH2 and 6M-PH4) showed lower stabilising effect, indicating that the effect is specific of the natural cofactor. Addition of dopamine also decreased the unfolding rate of TH, even at stoichiometric amounts, in agreement with the high affinity binding of the catecholamines. Additional experiments using the coupled *in vitro* cell free rapid transcription-translation system (RTS) for the synthesis of hTH1 in the presence or absence of BH_4 also revealed the stabilizing role of the cofactor. The stabilization might

also be caused by catecholamines, which production is stimulated upon increasing concentration. Nevertheless, the chaperone effect of the cofactor *per se* was proven by using specific AADC inhibitors. Furthermore, the mutant hTH1-L205P, associated to DRD and juvenile Parkinsonism is also stabilized by BH_4, revealing the potential of BH_4 as a natural chaperone to treat TH related disorders. Finally, we investigated the *in vivo* effect of BH_4 in wild type young adult mice supplemented with BH_4 at two different dose regimes. The cofactor was able to cross the BBB in a concentration dependent way. Moreover, a small but detectable and reproducible increase in TH activity and protein was observed, whereas the mRNA of TH was unchanged, further demonstrating a natural chaperone function for BH_4.

3.2.2. Stabilization of the AAAH by pharmacological chaperones (Paper IV)

Recently, Pey *et al.* identified four compounds (compounds I-IV) from a chemical library of organic molecules as stabilisers of PAH conformation, both *in vitro* and *in vivo*, suggesting its possible use as pharmacological chaperones for the treatment of PKU (Pey et al., 2008). In this paper we thus aimed to comparatively study the effect of these compounds on the three hydroxylases, to better unravel its specificity and therapeutic potential. As done earlier for human PAH (hPAH), the direct *in vitro* stabilization of human TH isoform 1 (hTH1) and human TPH2 (hTPH2) by compounds I-IV was analyzed by differential scanning fluorimetry (DSF). Compound I presented a small stabilising effect in hPAH, but no effect at all in the other two proteins. In contrast, compound II, with a small effect on hPAH and no effect in hTPH2, showed an important stabilising effect of hTH1. Interestingly, compound III strongly stabilised the three enzymes with similar up-shift of the melting temperature ($T_{0.5}$), whereas compound IV appeared specific for hPAH, with no effect on hTH1 and even a small destabilising effect on hTPH2. R202H-hTH1, an up to now uncharacterised variant of TH associated to DRD that shows decreased thermal stability ($T_{0.5}$~44 ^{o}C vs ~48 ^{o}C for the wt) suggesting a conformational defect, was also strongly stabilized by compounds II and III.

In vivo experiments were performed using wild type mice treated with 5 mg/kg/day of each compounds II-IV during 12 days. Compound II, which was an important and specific stabiliser for hTH1 *in vitro*, did not have any measurable effects in the mice. In contrast, compound III greatly stabilised PAH and TH, but not TPH, *in*

vivo, measured as a stimulation of total activity and protein. The effects of compound IV were surprising since a decrease in neurotransmitters and their metabolites was found, reflecting a possible destabilising effect, notably on TPH. In all conditions, levels of BH_4 and mRNA for the enzymes were normal and no changes were detected between control and treated samples.

All together our results point to the importance of comparative studies of ligand binding in proteins showing high structural and functional similarity, though different tissue localization, in order to rationally design specific drugs and avoid secondary harmful effects. Furthermore, the stabilizing effect of compound III on wt TH and the mutant R202H both *in vitro* and *in vivo* points to its potential as pharmacological chaperone for the treatment of TH-associated neurological disorders.

4. General discussion

4.1. The AAAH in *C. elegans*

4.1.1. Regulation of cePAH and ceTH

The low similarity at the regulatory domain of both PAH and TH reveals regulatory differences between these enzymes. In the case of PAH, the enzyme from the worm lacks the tight regulation exerted by the substrate (activation and positive cooperativity). In humans, both mechanisms ensure a fast degradation of L-Phe from the diet (Kaufman, 1986) since when accumulated in the bloodstream, this amino acid quickly goes to the nervous system inducing mental retardation (Kahler and Fahey, 2003). In *C. elegans*, on the other hand, a role of the enzyme in protection of the nervous system appears to be less important, since *pah-1* mutants do not present a PKU-like phenotype. Moreover, administration of extra L-Phe in the worm diet does not have any deleterious effect (Paper I). Absence of allosteric regulatory mechanisms in cePAH suggests that an anabolic function of PAH could be more important, in contrast with the major catabolic role of hPAH. We hypothesise that this switch in function is related to the development of nervous system complexity (Paper I). ceTH also presents important differences in regulation when compared with mammalian TH. Apart from the lack of the negative cooperativity for the cofactor, ceTH is not inhibited by either the substrate or the catecholamine end-products. These two are important mechanisms in hTH regulation (Kumer and Vrana, 1996). These facts could be reflecting a less tight regulation in the synthesis of catecholamine neurotransmitters through TH in relation with a simpler nervous system in lower eukaryotes (Vie et al., 1999; Neckameyer et al., 2005). Moreover, phosphorylation at Ser35 by PKA in ceTH does not appear to activate enzymatic activity, as it has been shown to be the case in hTH (Dunkley et al., 2004). How phosphorylation regulates the worm enzyme remains unclear, although it could be related to the interaction of ceTH with partners (e.g. 14-3-3, AADC, GTPCH) or to the subcellular localization of the enzyme (e.g. a distribution between cytoplasm and membrane fractions, such as the synaptic vesicles). Interactions of the enzyme have been reported to be regulated by phosphorylation in hTH (Kleppe et al., 2001; Halskau et al., 2009). ceTH appears then as an enzyme lacking, at least partially, some of the

regulatory mechanisms characteristic of the mammalian orthologs. It seems reasonable to hypothesize that ceTH does not need as strict regulation as hTH and behaves as a constitutive enzyme, with low but constant activity. This also seems to be the case for the epidermal isoform of TH from *D. melanogaster*, which -as ceTH- is resistant to dopamine inhibition and phosphorylation activation (Vie et al., 1999).

4.1.2. Function and molecular biology of cePAH and ceTH

Pah-1 knock-out animals lacked an organic molecule in the cuticle that we identified as a melanin-like compound in the family of pheomelanins (Nighswander-Rempel, 2006). Until the publication of this work it was unknown that *C. elegans* contained melanin, but several studies in other invertebrates (notably insects) have shown that melanin is present in their cuticular fraction (Kato et al., 2006; Ninomiya and Hayakawa, 2007). Moreover, in several invertebrates, such as insects or *Geodia cydonium* (Wiens et al., 1998), PAH appears to be the first enzyme in the synthesis of melanin. As a possible remainder of this primitive function, PAH is expressed in human skin cells (Schallreuter et al., 2005) and, moreover, PKU patients show a decrease in skin pigmentation, facts that some authors have related to a role of PAH in melanogenesis (Schallreuter et al., 2008). In mammals, melanin synthesis is catalyzed by tyrosinase (del Marmol and Beermann, 1996), that converts tyrosine into L-DOPA. Interestingly, the *C. elegans* genome owns four putative genes for tyrosinases, all expressed in hypodermis (Blaxter, personal communication). PAH would function as a previous step to tyrosinase, providing an extra pool of tyrosine.

The up regulation of SOD in the *pah-1* mutants points to a protective role of worm melanin against oxygen harmful radicals. Melanin has been demonstrated to be a very effective oxygen species scavenger (Simon et al., 2009), and its presence in the cuticle of the nematode, in contact with the oxidizing environment, further supports the antioxidant role of the compound isolated in this work.

Another possible implication of PAH is related to the structural integrity of the cuticle. cePAH has been previously related to the synthesis of di-tyrosine cross-links of collagens and cuticlins (Loer et al., 1999), structural proteins of the cuticle (Parise and Bazzicalupo, 1997; Yang and Kramer, 1999). The strong phenotype of the double mutant *bli-3:pah-1*, with severe cuticle abnormalities, suggests a role of PAH in cuticle

structure maintenance. If these abnormalities are due to the lack of di-tyrosine bridges itself, or if melanin is also implicated, remains unclear.

The function of ceTH has been much more studied than in the case of cePAH, since the role of TH in the synthesis of catecholamines is completely established, as it is the important neurotransmitter role of these biological amines for both vertebrates and invertebrates. *Cat-2* mutants, defective in TH activity, display an extremely low level of dopamine as well as mechanosensory impaired function (Sawin et al., 2000). However, the molecular organization of the *cat-2* gene has not been studied in detail so far. We here describe, clone and characterise a new isoform of ceTH, different to the two shorter isoforms reported in Wormbase. This novel ceTH c isoform seems to be more consistent, in terms of domain structure and organization, with the rest of THs from lower and higher eukaryotes. The resulting cloned enzyme is active and stable and our results strongly support that isoform c of ceTH is the enzyme being encoded *in vivo* by the *cat-2* gene, whereas isoforms a and b appear very unlikely. In the era of genomic annotations and sequencing, computational mistakes on the databases are not a rare event due to the immense amount of information, and experimental confirmation of the data seems essential.

4.2. Natural and pharmacological chaperones of the AAAH

PKU is one of the metabolic diseases recognized as a misfolding disease (Waters, 2003), where an unstable or partially unfolded version of the protein is target for degradation in the proteasome, with partially or completely lost of function. Natural and pharmacological chaperones are small organic molecules that help the protein to fold correctly and restore proper biological function (Martinez et al., 2008).

As we have pointed out in the introduction, the AAAH are functionally and structurally related enzymes. For this reason comparative studies of the three enzymes are necessary especially when considering putative therapies for one of the enzymes, since the therapy could easily influence the other activities.

4.2.1. Natural chaperones: the BH_4 case

The therapeutic importance of BH_4 is well established, not only for PKU (Levy et al., 2007)), but also for hypercholesterolemia (Cosentino et al., 2008), diabetes

mellitus (Nystrom et al., 2004) and cardiovascular diseases (Moens and Kass, 2007) among others. For this reason it is important to elucidate the effect of BH_4 supplementation on the rest of the AAAH.

Whether or not BH_4 is able to cross the BBB has been a controversial issue (Shintaku, 2002; Hyland, 2007). Our experiments on mice with BH_4 supplemented diets confirmed that BH_4 actually is able to reach the nervous system, although high doses are necessary (i.e. 100 mg/kg/day), compared with the lower doses used to treat PKU (10-20 mg/kg/day) (Sanford and Keating, 2009) or other human disorders nowadays. Our first conclusion seems that the actual doses used in the clinic are unlikely to interfere with nervous system enzymes, as TH or TPH.

We further investigated the putative chaperone effect of BH_4 upon TH, in comparison with the situation regarding PAH and some forms of mild PKU (Blau and Erlandsen, 2004). Our *in vitro* and *in vivo* results clearly point to a similar stabilization of TH by BH_4, as it was previously suggested in studies with *ptps* knock-out mice where the absence of the cofactor produced a complete loss of TH protein while TPH was not affected (Sumi-Ichinose et al., 2001). Moreover, the experiments performed in a RTS system with TH mutant isoforms implicated in DRD, showed that the chaperone effect of BH_4 is also effective for some of these mutations, such as hTH1-L205P (Ludecke et al., 1996) (Paper III), which opens the possibility of BH_4 as a therapy for DRD and Parkinson's related diseases.

4.2.2. Pharmacological chaperones for the treatment of AAAH related diseases

The importance of pharmacological chaperones to stabilise the AAAH enzymes was demonstrated by Pey *et al.* (Pey et al., 2008). In such work, organic compounds that increased the thermal stability of PAH were identified, and four (compounds I-IV; see full name and structure in paper IV) were suggested as possible therapeutic agents for the treatment of PKU. As it was the case for BH_4 (paper III), investigation about the effect of the compounds on the other hydroxylases appears essential. Even though compartmentalization of PAH in liver and TH and TPH in neuroendocrine system is an efficient first barrier for drug selectivity, some molecules will be able to reach all locations (as it is the case for BH_4) at similar concentrations, and our work (paper IV) is a good example of these concepts.

In vitro studies with compounds I-IV showed a different behaviour in the thermal unfolding of the hydroxylases. Compound III was the only one that bound and stabilised human PAH, TH and TPH2 in a similar way, whereas compounds II and IV seemed to be specific stabilisers for TH and PAH, respectively. No specific potential chaperone was found for TPH.

In vivo studies are a necessary step in the drug design process, although the results from these studies are not so straightforward to analyse and interpret. For instance, compound III that stabilized all AAAH *in vitro*, produced a clear increase in total protein and activity in both TH and PAH *in vivo*, but not in TPH. A different compartmentalization between catecholaminergic and serotonergic neurons in mouse brain could explain this different behaviour of compound III. Other explanations might be a more strict regulation and protection of TPH inside the neurons, in comparison with TH. A similar situation is found for BH_4 (paper III) where, despite the ability of the cofactor to reach the nervous system and stabilise TH enzyme, no significant effect was detected regarding TPH (unpublished results). Another inconsistent *in vitro/in vivo* effect was seen for compound II, which exerted a huge *in vitro* stabilisation on the hTH1 protein but no effect on TH in mice brains. We hypothesise a poor uptake of the compound in nervous system due to e.g. low lipophilicity (Josserand et al., 2006; Ballet et al., 2008), which might hinder crossing the BBB. In summary, we have seen that *in vivo* mechanisms for drug delivery, especially concerning the well protected nervous system, are complex and should be analysed carefully.

The results on the effect of the pharmacological chaperones on mutants involved in DRD in the case of TH and PKU in PAH, point to the possibility of using these compounds as putative therapeutic agents in some forms of the diseases ((Pey et al., 2008) and paper IV). More experiments with cellular cultures and, even better, transgenic animals models, expressing different mutations will be necessary to develop these drugs.

5. Conclusions and future perspectives

5.1. The study of the AAAH in *C. elegans*

C. elegans is a simple and widely used model organism in biology. We have been interested in the comparative characterization of the human and nematode enzymes of the AAAH family. We have focused on enzymatic activity and regulatory aspects, and have also investigated some features of the *in vivo* function of PAH and TH. This investigation using the nematode has provided important evolutionary information regarding the structure, function and regulation of the AAAH.

PAH resulted to be implicated in the synthesis of a new compound localized in the cuticle of the animal (paper I). The spectroscopic properties of this isolated molecule fitted well those of pheomelanin, but a more detailed characterization remains to be done. The use of mass spectrometry to analyse the purified melanin-like compound appears as a good strategy to obtain a more detailed chemical structure. Besides, more *in vivo* studies with wt and mutant worms are being planned to get further knowledge into the physiological function of this newly identified compound. Since the increase of antioxidant enzymes and survival in the *pah-1* knock-out animals pointed to an oxygen radical scavenger role for the PAH end product, future experiments will aim to investigate that possibility.

As pointed out in the introduction TH is a well regulated enzyme (Kumer and Vrana, 1996), but there is no enough *in vivo* information about enzymatic regulation. We have characterised the recombinant enzyme from the nematode (paper II), showing that some regulatory mechanisms are conserved between humans and worms. We are very interested on studying how ceTH is regulated *in vivo* and how this regulation affects behaviour in *C. elegans*. Dopamine has been shown to be implicated in several complex behaviours of the nematode, as motility or egg-laying, and it would be a challenge to investigate how regulation of the enzyme is reflected in some of these physiological functions. We intend to make use of the customary *C. elegans* methodology, as GFP expression constructs, measurement of calcium currents in dopaminergic neurons, and diverse phenotypic analysis to investigate coupling of dopamine synthesis and transport to synaptic vesicles (Cartier et al., 2010), as well as a putative role of phosphorylation in subcellular localization.

5.2. Pharmacological chaperones for the AAAH

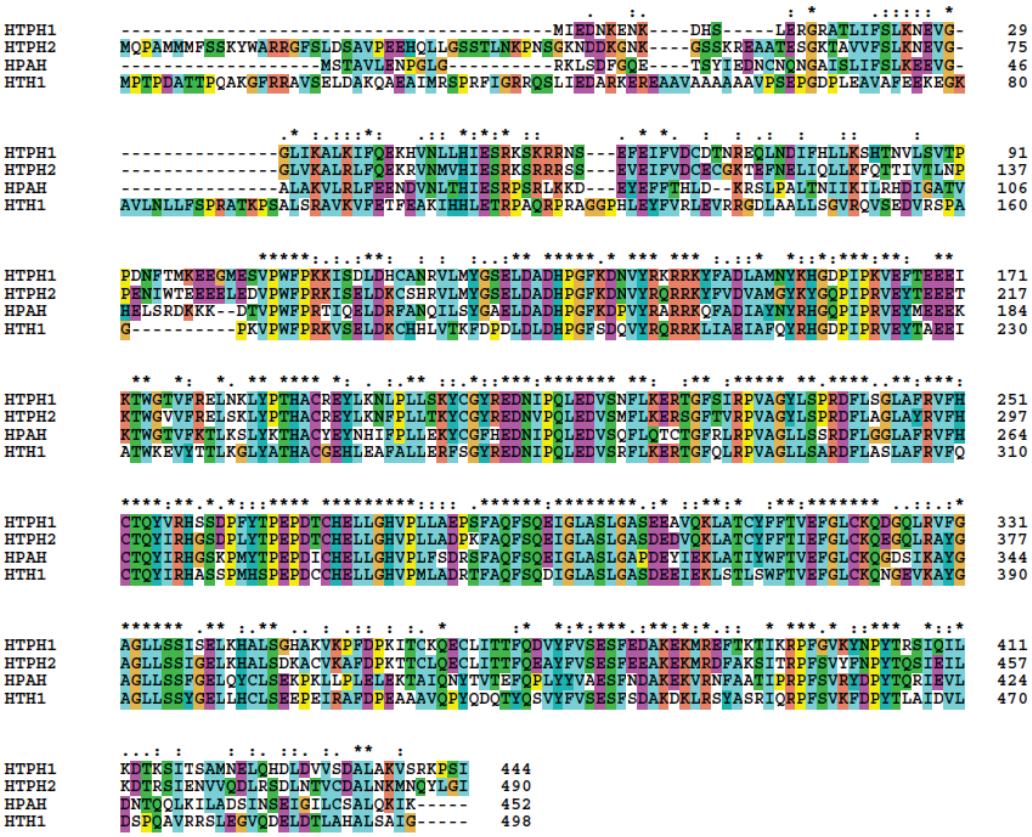
A HTP screening of a chemical library was carried out in the case of PAH to identify several compounds that increase protein stability and function as putative pharmacological chaperones (Pey et al., 2008). Four positive hit compounds were selected for further experiments, and we tested these compounds in parallel in PAH, TH and TPH2, revealing important information about specificity in protein stabilization of the three related enzymes by chaperone molecules (paper IV). A new HTP screening for TH and both TPHs seems an interesting next step in our investigation, in order to find new and specific stabilisers for these neuroendocrine enzymes, always keeping in mind the molecular properties of the compounds that facilitate crossing the BBB.

Finally, it would be interesting to perform experiments in mammalian cell cultures with several DRD related TH mutants (Knappskog et al., 1995; Ludecke et al., 1996; van den Heuvel et al., 1998). *In vitro* studies, both with BH_4 and the pharmacological chaperones (papers III and IV), showed that some of these mutations present impaired protein stability. Thus, the use of small compounds that increase stability is certainly a promising therapeutic alternative. Initial experiments in our laboratory with PC12 cultures supplemented with BH_4 and the compounds did not provide optimal conditions for reliable results, but improvement of cellular culture experiments constitutes an interesting field to study mutant-related stability therapies. Moreover, the possibility of expressing specific disease-mutant proteins in *C. elegans* opens the possibility to easy and inexpensive animal studies in our laboratory.

6. Appendix

6.1. Sequence alignment of the AAAH

Sequence alignment of the human aromatic amino acid hydroxylases, TPH1 (SWISS-PROT P17752), TPH2 (TrEMBL Q8IWU9), PAH (SWISS-PROT P00439) and TH (splicing isoform 1; SWISS-PROT P07101). The asterisks above the sequences indicate identity, while homologue properties of the residues are indicated with two dots or one dot, depending on the degree of similarity.



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