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TRANSCRIPTIONAL REGULATION OF TRYPTOPHAN HYDROXYLASE-2

by

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a Juanca y Ana,
por la paciencia

ABSTRACT

Dysregulation of serotonergic neurotransmission is a contributing cause of numerous pathologies. In the brain, the enzyme catalyzing the rate-limiting step in serotonin biosynthesis and thus controlling serotonin levels is tryptophan hydroxylase-2 (TPH2). The objective of this project is to study the regulation of TPH2 transcription by characterizing its promoter region.

Study of the 5' flanking region of the human TPH2 gene by means of reporter gene assays resulted in the finding of a core promoter and a repressing element between positions -179 and -88 relative to the transcription start site. In addition, the TPH2 promoter could be activated by Ca^{++} mobilization in a cell-line model of serotonergic neurons, but not in other cell lines. In agreement, Ca^{++} mobilization in this model also induced endogenous transcription of TPH2.

This work is the first one to identify the promoter region of the TPH2 gene and to report its activity-dependent regulation of transcription.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamin, serotonin
5-HTP	5-hydroxytryptophan
5-HTT	5-HT transporter
5'-RACE	5' Rapid amplification of cDNA end
A	Adenine
AAAD	Aromatic amino acid decarboxylase
ADHD	Attention deficit-hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AP-1	Activating protein-1 / Adaptor primer-1
AP-2	Activating protein-2 / Adaptor primer-2
ATF-1	Activating transcription factor-1
BA	Brocca's area
BAPTA-AM	1,2-bis-(o-aminophenoxy)-ethane-N,N,-N',N'-tetraacetic acid tetraacetoxy-Methyl ester
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BH_4	Biotetrahydropterin
Bp	Base pair(s)
BP	Bipolar disorder
BRE	TFIIB-recognizing element
bZIP	Basic region leucine zipper
C	Cytosine
CaM	Calmodulin
CaMK	Ca^{++}-calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CaRE	Calcium-responsive element
CBP	CREB-binding protein

C/EBP	CCAAT/enhancer binding protein
cDNA	Complementary DNA
CDS	Coding sequence
ChIP	Chromatin immunoprecipitation
CICR	Ca^{++}-induced Ca^{++} release
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CREM	CREB modulator
CREST	Ca^{++}-responsive transactivator
CRPG	Chlorophenol red β-D-galactopyranoside
CSF	Cerebrospinal fluid
DA	Dopamine
DEPC	Diethylpyrocarbonate
DHP	Dihydropyridine
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DOPA	3,4-dihydroxy-L-phenylalanine
DPE	Downstream promoter element
DREAM	Downstream regulatory element-antagonist modulator
DRN	Dorsal raphe nucleus
E	Estrogen
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPM	Elevated plus maze
ER	Endoplasmic reticulum / Estrogen receptor
FBS	Fetal bovine serum
fMRI	Functional magnetic resonance imaging

Freud-1	5' repressor element under dual repression binding protein-1
G	Guanine
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GTF	General transcription factor
HAT	Histone acetyltransferase
HBSS	Hank's Balanced Salt Solution
HDAC	Histone deacetylase
HEK 293	Human embryonic kidney 293
HPA	Hypothalamus-pituitary-adrenal
IEG	Immediate-early gene
Inr	Initiator
IP_3R	1,4,5-inositol triphosphate receptor
ISH	*in situ* hybridization
Kb	Kilobase pairs
KID	Kinase inducible domain
KO	Knock out
LD	Linkage disequilibrium
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
MDD	Major depressive disorder
β-ME	β-mercaptoethanol
MeCP2	Methyl-CpG binding protein-2
MEF-2	Myocyte-enhancer factor-2
M-MLV	Mouse-Moloney murine leukemia virus
mRNA	Messenger RNA
MRN	Median raphe nuclei
MTE	Motif 10 element

MW	Molecular weight
NA	Noradrenaline, norepinephrine
NCBI	National Center for Biotechnology Information
NGF	Nerve growth factor
NeuroD2	Neurogenic differentiation-2
NFAT	Nuclear factor of activated T cells
NF-Y	Nuclear factor-Y
NMDA	N-methyl-D-aspartate
NRSF	Neural-restrictive silencer factor
OCD	Obsessive-compulsive disorder
PAH	Phenylalanine hydroxylase
PBS	Phosphate buffer and saline
PC12	Pheochromocytoma 12
pCREB	PhosphoCREB
pCPA	para-chlorophenylalanine
PEA	Phenyletyilamine
PET	Positron emission tomography
PFC	Prefrontal cortex
Pit-1	Pituitary specific transcription factor-1
PKA	Protein kinase A
PLC	Phospholipase C
PR	Progestin receptor
RE-1	Repressor element-1
REST	RE-1 silencing transcription factor
RPA	RNAse protection assays
rpm	Revolutions per minute
RSK	Ribosomal S6 kinases
RT-PCR	Reverse transcription-polymerase chain reaction
RyR	Ryanodine receptor
SIDS	Sudden infant death syndrome
SNP	Single nucleotide polymorphism

SSRI	Selective serotonin reuptake inhibitor
T	Thymine
TAF	TBP-associated factors
TBP	TATA-box binding protein
TESS	Transcription element search system
TF	Transcription factor
TFBS	Transcription factor binding sites
TFIID	Transcription factor IID
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
TRH	Thyrotropin-releasing hormone
trk	Tyrosine kinase
TSS	Transcription start site
USF	Upstream stimulatory factor
UTR	Untranslated region
VGCC	Voltage-gated Ca^{++} channel
VMaT	Vesicular monoamine transporter

CHAPTER I – INTRODUCTION

1.1 The serotonergic system

Serotonin (5-HT) was originally discovered as a vasoconstrictor substance present in blood serum. Although this property was noticed for the first time in 1868, only eighty years later the compound was identified as the biogenic amine 5-hydroxytryptamine [reviewed in Rapport, 1997]. Initial investigations focused on cardiovascular and gastrointestinal actions of the new substance but the finding that 5-HT was abundant in the brain and presumably involved in major psychoses sparked the interest in its role in the central nervous system (CNS). Eventually, 5-HT was established as a central synaptic neurotransmitter [reviewed in Jacobs and Azmitia, 1992].

1.1.1 Anatomy and functions

The serotonergic system in mammals arises from the brainstem and projects to most regions in the central nervous system. The cell bodies are located to the sides of the midline following the rostrocaudal axis of the brainstem, but also appear in the reticular formation [Törk, 1990]. The serotonergic neurons were originally localized by histochemical fluorescence and grouped into nine nuclei (B1 to B9), that mostly overlapped the raphe nuclei [Dahlström and Fuxe, 1964]. These nuclei can be located either in the rostral division (in the midbrain and the rostral

pons) or the caudal division, extending from the caudal pons into the medulla oblongata. Importantly, the rostral division contains 85% of all serotonergic neurons in the brain and includes the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN), among other smaller nuclei [Hornung, 2003].

The raphe nuclei also include non-serotonergic cells, that express a variety of neurotransmitters and neuropeptides, such as dopamine (DA), noradrenaline (NA), γ-aminobutyric acid (GABA), glutamate, enkephalin, substance P, thyrotrophin-releasing hormone (TRH) and others [Jacobs and Azmitia, 1992]. Cellular heterogeneity is even seen within the serotonergic neurons, since these can be diverse in terms of shape, size, orientation [Baker et al, 1990] and pharmacological and electrophysiological responses [Marinelli et al, 2004; Beck et al, 2004].

The serotonergic fibers, sprouting from relatively few cells, arborize richly and connect to the entire CNS through extensive and diffuse projections and collateral branches that do not always make synaptic contacts. Fibers stemming from the rostral group innervate most of the cerebral cortex, limbic system, diencephalon and basal ganglia, while projections from the caudal division reach brainstem nuclei and the spinal cord [Hornung, 2003]. Some areas, like the brainstem and the cerebellum receive afferents from both divisions [Törk, 1990]. Afferents to serotonergic neurons come mainly from the raphe nuclei themselves, but also from the limbic forebrain, the hypothalamus, the locus coeruleus and dopaminergic regions such as the substantia nigra and the ventral tegmental area [Jacobs and Azmitia, 1992]. Importantly, the serotonergic projections are

topographically organized, each nucleus innervating specific targets. This organization is extended within each nucleus, where distinct subpopulations show specific connections and therefore unique functional properties [Abrams et al, 2004].

Development of the serotonergic system has been studied in several species, including rodents, nonhuman primates and human. During early gestation, two separate clusters of neurons arise from the ventral hindbrain, one rostral and one caudal. In the human, the first serotonergic neurons appear during week 5 and the raphe nuclei are defined by week 15 [reviewed in Whitaker-Azmitia, 2001]. In the rat, 5-HT immunoreactivity can be seen in the rostral group at embryonic day 12 (E12), whereas the caudal group becomes 5-HT positive two days later. Immediately after, fibers start to sprout reaching target regions at various times during development or postnatally [Rubenstein, 1998; Jacobs and Azmitia, 1992]. At the molecular level, several factors have been found to be necessary for 5-HT cell fate specification, such as Lmx1b, Nkx2.2 and Pet-1, the latter being the only one specific to serotonergic neurons [Hendricks et al, 1999].

The serotonergic system is involved in the modulation of an impressive number of functions. To begin with, 5-HT plays a developmental role as a trophic factor, regulating the maturation of target areas [Whitaker-Azmitia, 2001]. Central serotonin also acts on autonomic nuclei that control, for example, cardiac [Jordan, 2005] or sexual function [Giuliano and Clement, 2005]. By targeting the hypothalamus, 5-HT regulates endocrine activity, such as that of the

hypothalamus-pituitary-adrenal (HPA) axis [Lowry, 2002]. In addition, 5-HT is related to the modulation of physiological features like nociception, sleep-wake-arousal cycles, sexual behavior and food intake. Finally, the impact of 5-HT can also be seen in more complex behavioral traits, such as aggression, fear and anxiety [Jacobs and Azmitia, 1992].

1.1.2 Serotonergic neurotransmission

The serotonergic neuron can synthesize, store, release upon stimulation, reuptake and degrade 5-HT. 5-hydroxytryptamine is synthesized in the cytosol of serotonergic neurons in a two-step reaction starting off from L-tryptophan, an essential amino acid (Figure 1.1). In the first, rate-limiting step, L-tryptophan is converted to 5-hydroxytryptophan (5-HTP). In neurons, this reaction is catalyzed by the enzyme tryptophan hydroxylase-2 (TPH2), while in other (peripheral) serotonin-producing cells the reaction is carried out by a different isoform, tryptophan hydroxylase-1 (TPH1) [Walther and Bader, 2003]. The second step, catalyzed by the enzyme aromatic amino acid decarboxylase (AAAD), consists of the decarboxylation of 5-hydroxytryptophan into 5-hydroxytryptamine. While the first step is specific to 5-HT production, the second is common to the biosynthesis of other monoamine neurotransmitters such as dopamine or noradrenaline [Kandel et al, 2000].

Cytosolic 5-HT can be metabolized by monoamine oxidases (MAO) into 5-hydroxyindoleacetaldehyde, which in turn can be oxidized to 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT. There are two isoforms of MAO,

named A and B, of different but overlapping specificity. 5-HT, as well as noradrenaline (NA), are metabolized preferentially by MAO A, whereas MAO B has been found to oxidize phenyletyilamine (PEA) and both isoenzymes degrade dopamine (DA) [Shih et al, 1999]. Interestingly, serotonergic neurons have more MAO B than MAO A, possibly to prevent the excessive incorporation of other substrates, like DA. Alternatively, cytosolic serotonin can be transported into vesicles by the vesicle monoamine transporter-2 (VMaT2). Upon stimulation, the serotonergic neuron depolarizes and the vesicles fuse to the membrane and release 5-HT into the extracellular milieu. At this point, 5-HT binds to a specific receptor, diffuses away or is taken up back into the pre-synaptic neuron by the 5-HT transporter (5-HTT), a 12-transmembrane-domain protein homologous to NA and DA transporters [Kandel et al, 2000].

There exist fifteen 5-HT receptors, divided in seven classes based on structural and operational differences [Hoyer and Martin, 1997]. Except for the 5-HT3 subtype, a ligand-gated ion channel, the receptors are metabotropic. Different receptors are coupled to different G-proteins, leading to a diverse set of intracellular responses, such as inhibition or stimulation of cAMP, activation of phospholipase C (PLC) and modulation of K^+ and Ca^{++} channel activity [Barnes and Sharp, 1999]. These receptors usually act as post-synaptic targets of 5-HT, but can also work as pre-synaptic autoreceptors, as is the case of somatodendritic 5-HT1A and nerve-terminal 5-HT1B. Importantly, activation of these receptors can lead to changes in neuronal firing, and 5-HT synthesis, turnover and release [Piñeyro and Blier, 1999; Barton and Hutson, 1999].

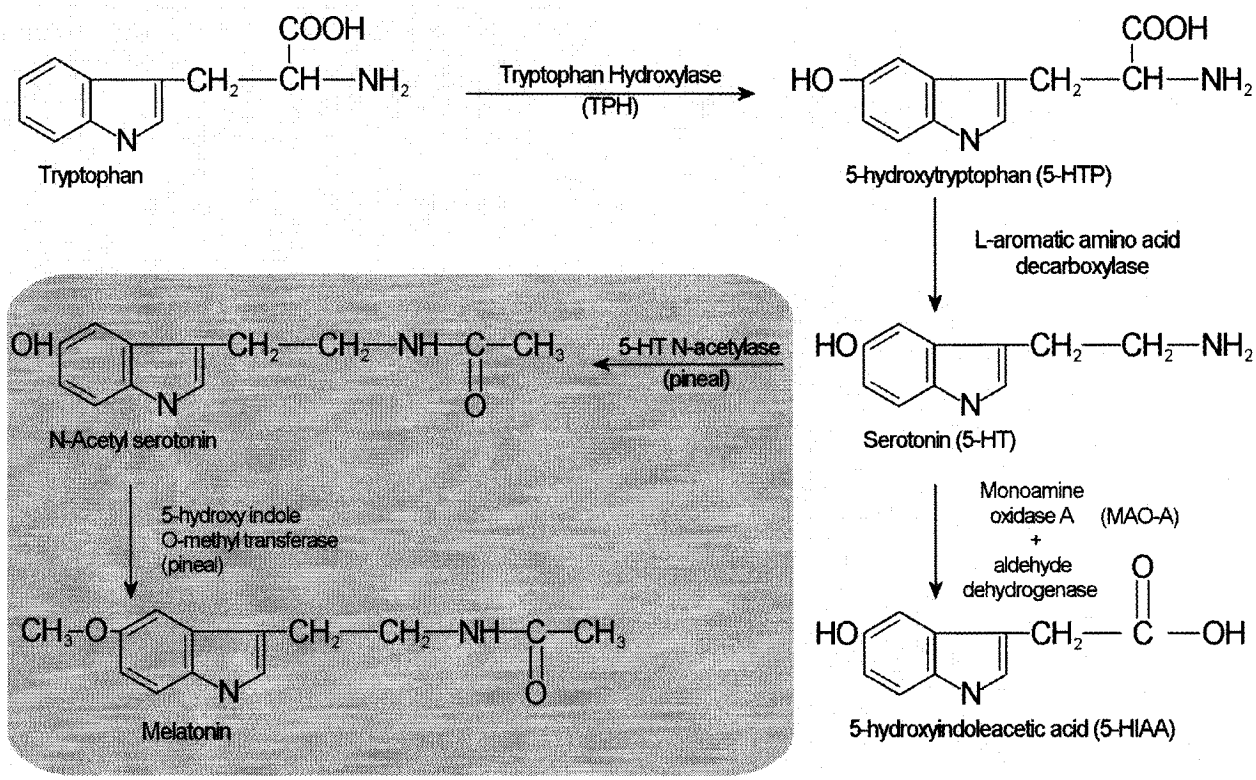

Figure 1.1 – Biosynthesis and metabolism of serotonin

1.1.3 Evidence of association between serotonin and mental illness

Consistent with its vast array of functions, the serotonergic system has been related to numerous diseases, specially, but not limited to, pathological behavior and psychiatric disorders. Different lines of evidence point to serotonergic dysfunction as a contributing cause of pathologies such as depression, bipolar disorder, anxiety disorders, suicidal and impulsive behavior, autism and many others.

Unipolar depression, also called Major Depressive Disorder (MDD), is a chronic disease, partially heritable, characterized by alterations in the emotional, cognitive and psychomotor aspects of behavior. The course of the illness is variable, and so are the symptoms and response to medication [Blier, 2006]. For this reason, MDD is recognized as a complex, heterogeneous syndrome for which the pathogenesis is not well understood. However, there is experimental support for the involvement of serotonin dysfunction in the etiology of depression. To begin with, acute tryptophan depletion causes mood lowering in susceptible healthy subjects and relapse in formerly depressed patients, particularly the ones that took selective serotonin reuptake inhibitors (SSRI) [van der does, 2001; Bell et al, 2005]. Secondly, all current antidepressant treatments affect central 5-HT levels, like electroconvulsive shock, MAO inhibitors, tricyclics and SSRIs. In particular, SSRIs target specifically the 5-HTT. Interestingly, different SSRIs do not share structural or functional properties, except for their capacity to block the 5-HTT [Vaswani et al, 2003]. In addition, expression levels and binding to 5-HTT as well as other components of the serotonergic system have been found altered in the

brains of depressed patients [Arango et al, 2002]. Bipolar disorder (BP), a related disease that alternates between manic and depressive states and is effectively treated with SSRIs, also belongs to the group of 5-HT-related mental disorders.

Anxiety disorders, including generalized anxiety disorder, panic attacks, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder are characterized by elevated fearfulness, which translates into diverse organismic as well as psychiatric symptoms. These symptoms can be treated with SSRIs, suggesting a role for 5-HT, that is further supported by independent evidence. In panic disorder, for instance, challenge and treatment studies, as well as brain-imaging, supported the inhibitory influence of 5-HT on panic [Maron and Shlik, 2006]. In OCD, patients given 5-HT2 receptor agonist meta-chlorophenylpiperazine suffered an exacerbation of their symptoms [Zohar et al, 2000]. In addition, a general role of 5-HT in anxiety has been further supported by a series of elegant studies showing how disruption of 5-HT1A receptor gene can cause anxiety in animal models [reviewed in Gross and Hen, 2004].

Numerous psychiatric disorders, such as MDD, BP, substance abuse and schizophrenia, constitute a prominent risk factor of suicidal behavior, that is, the attempt and possible completion of suicide [Joiner et al, 2005]. Suicidal behavior, a heritable trait, has been related to 5-HT hypofunction, although this relation appears to be independent of the co-morbid illness [Mann, 2002]. Suicide attempters show a blunted prolactin response in fenfluramine challenge studies, an indication of diminished post-synaptic 5-HT action [Joiner et al, 2005]. In addition, serious suicide attempters present lower plasma and cerebrospinal fluid (CSF) levels of 5-HIAA, as well as reduced serotonergic function in the ventral prefrontal

cortex, an area associated with impulse inhibition [Mann, 2002]. Interestingly, suicidal behavior has been highly linked to impulsivity and aggression [Turecki, 2005], two personality traits that are known to be modulated by 5-HT function [Evenden, 1999; Popova, 2006]. For instance, among other evidence, knocking out MAO A, the isoform that preferentially degrades 5-HT, led to aggressive mice [Shih and Chen, 1999].

Autism is a complex neurodevelopmental disorder featuring symptoms like stereotypic behavior and impairments in social interaction, language, communication and imaginative play. The pathogenesis of autism is yet undetermined, but it is known that it has a high familial heritable component. Evidence of serotonin involvement includes the use of SSRIs to improve certain behavioral symptoms and a well established increase of 30% in 5-HT platelet levels in autistic patients [Chugani, 2002]. Alternatively, the role of 5-HT in brain development has raised the hypothesis that pre-natal serotonergic dysfunction is at the core of autism [Chugani, 2002; Scott and Deneris, 2005].

There are other pathologies that have been related to central 5-HT function, but are not psychiatric in nature. For example, migraine, where 5-HT1B receptor agonists are used as medication [Buzzi and Moskowitz, 2005], or sudden infant death syndrome (SIDS), in which as much as 50% of the cases have been shown to present developmental defects in the medullary 5-HT system [Kinney, 2005].

The majority of these disorders are still poorly understood, yet all this accumulated evidence underscores the importance of understanding the extent and nature of the influence of any serotonergic dysfunction. Whether in normal or

pathological state, further investigation on the various components of serotonergic neurotransmission is guaranteed to contribute to devise or improve current therapies.

1.2 Tryptophan hydroxylation

The synthesis of serotonin is a key step in serotonergic neurotransmission, since it determines the amount of neurotransmitter available in the serotonergic cell. Availability of neurotransmitter can influence the amount of 5-HT released in one depolarization event, as well as the sustainability of the release over a series of action potentials. Since the rate-limiting step in the biosynthesis of 5-HT is catalyzed by tryptophan hydroxylase, this enzyme is a major candidate to constitute a regulatory checkpoint of serotonergic output, which, when defective, can be a contributing cause of 5-HT-related illness. For these reasons, TPH has been investigated since the discovery of serotonin.

1.2.1 Early findings in tryptophan hydroxylation

Tryptophan hydroxylation is the conversion of the essential amino acid L-tryptophan into 5-hydroxytryptophan. The enzymatic catalysis of this reaction was detected for the first time in the microorganism *Chromobacterium violaceum* [Mitoma et al, 1955]. Interestingly, this hydroxylation was not a step toward the synthesis of 5-HT, but rather to violacein, a purple pigment. Thereafter, this activity

was detected in a variety of tissues from different species, as listed by Grahame-Smith, such as mouse mast cell tumor, rat and guinea pig intestinal mucosa and carcinoid tumor [Grahame-Smith, 1964]. The work by Grahame-Smith, who studied rabbits and dogs, was especially important because it measured enzymatic hydroxylation of tryptophan in the brain for the first time [Grahame-Smith, 1964]. Since 5-HT is hydrophilic and cannot cross the blood-brain barrier (BBB) in substantial amounts, this finding was largely expected. TPH activity was first detected in the brainstem, but subsequent reports also found it in other brain regions innervated by serotonergic fibers.

The tryptophan hydroxylating activity from brain, mastocytoma and pineal extracts was soon recognized to be distinct from the activity of phenylalanine hydroxylase (PAH), a liver enzyme that can also hydroxylate L-tryptophan [Hosoda and Glick, 1966]. Both enzymes, together with tyrosine hydroxylase (TH) belong to the aromatic amino acid hydroxylases family. They require O_2 and tetrahydrobiopterine (BH_4) as co-substrates and Fe^{++} as necessary cofactor, although the optimal pH and substrate affinities are different [Fitzpatrick, 1999]. TPH was classified as an oxidoreductase and formally denominated tryptophan-5-monooxygenase, or EC 1.14.16.4.

Early on, TPH appeared as the rate-limiting enzyme in the synthesis of 5-HT [Lovenberg et al, 1967]. In support of this idea, 5-HTP is detectable in the brain only in trace amounts [Long et al, 1982], indicating that the more ubiquitous AAAD is also more active in serotonergic cells. As expected, TPH activity was found to parallel 5-HT levels. In the rat brain, 5-HT levels at different stages of pre- and post-natal development changed following changes in TPH activity levels [Deguchi

and Barchas, 1972]. Furthermore, the same article reported a correlation between 5-HT levels and TPH activity in different brain regions in the adult rat. Irreversible inhibition of TPH activity with para-chlorophenylalanine (pCPA) led to diminished 5-HT levels [Koe and Weissman, 1966], and lesion on the midbrain raphe nuclei led to reduction in the same proportion of forebrain 5-HT levels and TPH activity [Kuhar et al, 1979].

1.2.2 Evidence towards two tryptophan hydroxylase isoforms

The protein responsible for the tryptophan hydroxylation activity was difficult to purify, as reported by several early attempts, due to its tendency to aggregate into polymers and its very low expression [reviewed in Kappock and Caradonna, 1996]. In 1975, Joh and coworkers, after pooling brainstems from 260 rats, purified TPH and produced a specific antibody [Joh et al, 1975], which confirmed that TPH protein was present in serotonergic neurons.

The purification of TPH from various sources, mainly brainstem and pineal gland, but also mast cells and intestinal enterochromaffin cells, led to a more detailed study of its molecular and kinetic properties. On the one hand, these studies confirmed well-known facts about TPH, such as its dependency on reduced pterines and its ability to hydroxylate phenylalanine.

On the other hand, results from purified extracts showed discrepancies that suggested the existence of two TPH isoforms. TPH was consistently found to form soluble homotetramers, but the molecular weights (MW) reported were 53 or 59 kDa depending on the protein source. Similarly, Stokes radius measured by gel

filtration also depended on the source. Interestingly, larger MW and radius were found in the brainstem as opposed to mastocytoma protein [reviewed in Kappock and Caradonna, 1996]. Kinetic and biochemical properties such as K_M, V_{max} and isoelectric point differed in various reports depending on the analyzed tissue [reviewed in Walther and Bader, 2003]. Finally, antibodies raised to TPH presented inconclusive results. For instance, a monoclonal antibody recognized a 53 kDa protein in pineal gland samples, but a 56 kDa protein from dorsal raphe extracts [Haycock et al, 2002].

The hypothesis of a second isoform was put forward a number of times to explain the variant results, particularly between pineal and brain extracts [Grahame-Smith, 1964; Ichiyama et al, 1976]. However, the cloning of transcripts carrying the same coding sequence (CDS) from pineal gland and brainstem buried the two-isoforms hypothesis for some time. In 1986, Darmon et al reported the partial cloning of rat pineal TPH [Darmon et al, 1986], a work completed in 1988 with the cloning of two full-length cDNAs, that differed only in the 3'-UTR [Darmon et al, 1988]. Subsequently, human TPH was cloned from a serotonin-secreting carcinoid tumor. The clone presented a 1332-bp-long CDS, which encoded a 51 kDa protein that was more than 90% homologous to the rat one [Boularand et al, 1990]. TPH cDNA from rabbit pineal gland, also highly homologous to the rat one, was shown to hybridize to rabbit brainstem RNA under stringent conditions [Grenett et al, 1987]. What is more, cDNA cloned from rat brainstem was identical to the one previously cloned from pineal gland [Kim et al, 1991], somewhat settling the dispute over a second TPH gene.

The isoform originally cloned was TPH1, whose mRNA is abundant in the pineal gland but about 150 times scarcer in the brainstem, thus detection by Northern blot or ribonuclease protection assays required pooling five to ten brainstems [Dumas et al, 1989]. Since available TPH antibodies detected both isoforms, protein levels in pineal gland and raphe appeared to be of similar magnitude. This discrepancy was explained by findings that gave raise to complicated putative regulatory hypotheses. Briefly, TPH transcripts differing in their UTR were differentially expressed in pineal gland and the raphe [Dumas et al, 1989]. These authors also found that the raphe variant was more efficiently translated *in vitro*, further suggesting that differences in TPH expression were due to post-transcriptional tissue-specific regulation.

Nevertheless, this evidence was unconvincing to explain the high raphe TPH activity coupled to trace amounts of TPH transcript. The puzzle was finally solved in 2003 when Walther and colleagues reported the discovery of a second gene encoding a neuronally-restricted tryptophan hydroxylating enzyme [Walther et al, 2003].

1.3 Neuronal tryptophan hydroxylase (TPH2)

The gene for the second tryptophan hydroxylase isoform was discovered after finding normal levels of brain 5-HT in mice genetically deficient for the known TPH isoform. Using the published TPH sequence, Walther and coworkers knocked out TPH1 and expected a lethal phenotype. Surprisingly, they obtained viable

homozygous pups that had only trace amounts of 5-HT in duodenum, lacked it completely in whole blood and pineal gland, but had normal levels in hippocampus and frontal cortex. What is more, the TPH1-/- mice performed normally in behavioral tests like the elevated plus maze (EPM) and hole board. After screening the human genome with short translated TPH sequences, the new gene was discovered and immediately cloned from mouse, rat and human [Walther et al, 2003].

1.3.1 TPH2 gene and protein structure

For TPH2, human, rat and mouse share the same gene structure, which consists of eleven exons, while the sequences at the nucleotide and the amino acid levels are highly homologous. The human gene, TPH2, is reported to produce a 2350-bp-long transcript that encodes a 490-amino acid protein (56 kDa). The rat gene, or Tph2, produces a 2581-bp transcript that translates into a 485 amino acid protein (55.6 kDa). Similarly, the mouse gene, also designated Tph2, transcribes into a 2638-bp-long mRNA that predicts a protein of 488 amino acids or 55.8 kDa [Walther et al, 2003]. In humans, the gene is located in chromosome 12q21.1, where it spans 93.6 kb, while it is located in 7q22 in the rat (109.7 kb) or chromosome 10 D2 in the mouse (106.4 kb) [http://www.ncbi.nlm.nih.gov].

TPH2 has also been detected in chicken, chimpanzee, zebrafish, torafugu [Zhang et al, 2005b] and macaque, where the amino acid homology to human TPH2 raises to more than 98% [Chen et al, 2006].

The sequence of TPH2 places it in the superfamily of aromatic amino acid hydroxylases, along with its paralogs PAH, TH and TPH1 [Fitzpatrick, 1999]. Importantly, TPH2 is very similar to TPH1, presenting a homology at the protein level of 71% in humans, 68% in rats and 70% in mice [Walther et al, 2003; Walther and Bader, 2003]. These similarities were enough so that commercially available antibodies recognized both isoforms [Walther et al, 2003], and only recently isoform-specific peptide antibodies have been reported [Sakowski et al, 2006b]. There is, however, a notable difference in the N-terminal region, which is around fifty amino acids longer in TPH2. Investigation of the amino acid sequences showed that important amino acids characterized in TPH1 were conserved, such as (a) phosphorylation sites for Ca^{++}-calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA) at serine residues 104 and 306, respectively; (b) leucine zipper motif at the C-terminal, (c) hydrophobic interaction domain in the N-terminal and also (d) several residues involved in the binding of tryptophan, BH_4 or Fe^{++} [reviewed in Walther and Bader, 2003].

To test these predictions, TPH2 has been expressed in heterologous systems, like *Escherichia coli*, usually fused to maltose-binding protein (MBP) [McKinney et al, 2005; Ogawa and Ichinose, 2006]. Recombinant TPH2 showed tryptophan hydroxylation activity *in vitro*. However, as expected from the distinct properties of pineal and brain extracts, TPH2 differed from TPH1 in the kinetic properties. Human TPH2 presented higher substrate specificity than TPH1, showing a higher preference for tryptophan over phenylalanine than TPH1. Secondly, overall tryptophan hydroxylating activity, as measured by V_{max} and K_M,

was lower for TPH2, maybe due to more stringent substrate recognition [McKinney et al, 2005].

On the other hand, recombinant TPH2 has shown insolubility, tendency to aggregate and susceptibility to oxidative damage similar to TPH1 [McKinney et al, 2005; Carkaci-Salli et al, 2006]. Homopolymerization in tetramers, as seen in TPH1, has been repeatedly observed for human [McKinney et al, 2005] or mouse TPH2 [Sakowski et al, 2006a]. Interestingly, mutants lacking the 24-aa-long C-terminal that carries the putative polymerization region, did not form tetramers [Carkaci-Salli et al, 2006]. Conversely, mutation of Pro447 to Arg in mouse Tph2 (a naturally occurring variant), did not affect the formation of tetramers [Sakowski et al, 2006a].

In terms of phosphorylation, human TPH2 accepted three phosphates per protein molecule *in vitro*. One of the phosphorylation sites was identified at Ser19, a residue absent from TPH1 that is analogous to Ser40 in the N-terminal regulatory region of TH. Phosphorylation of Ser19 or the other sites allow the binding of 14-3-3 protein. Different 14-3-3 isoforms constitute a family of proteins that is widely expressed in the brain and bind phosphoproteins, often influencing activity and stability [van Heusden, 2005]. However, neither the phosphorylation alone nor the 14-3-3 binding increased TPH2 activity *in vitro* [McKinney et al, 2005].

Taken together, the studies on the protein aspects of TPH2 confirmed its tryptophan hydroxylation function and its similarities to TPH1, but stressed that regulatory mechanisms like phosphorylation or substrate affinity are distinct, suggesting properties specific to the needs of the CNS.

1.3.2 Tissue-specific expression of TPH2 and TPH1

The restricted tissue-specific gene expression of TPH2 is one of its most notable characteristics. TPH2 expression has now been investigated in different tissues of mouse, rat, macaque and human at the mRNA level, mainly by *in situ* hybridization (ISH) and RT-PCR. Investigation of TPH protein distribution by immunoreactivity has lagged behind because isoform-specific antibodies have only recently begun to emerge [Sakowski et al, 2006b].

As revealed by Walther et al, Tph2 expression appeared to be only neuronal since the mRNA could not be detected by RNAse protection assays (RPA) in kidney, lung or duodenum but showed a strong signal in the mouse brainstem, where it was about 150 times higher than Tph1 [Walther et al, 2003]. Plenty of evidence supports Tph2 expression in the rodent raphe nuclei. Expression of Tph2 mRNA in the brainstem was confirmed by RT-PCR in mouse [Kim et al, 2005] and rat [Zhou et al, 2005b]. Subsequently, the anatomical location was narrowed to the raphe nuclei by ISH in mouse [Côté et al, 2003; Clark et al, 2005] and rat [Patel et al, 2004; Malek et al, 2005]. Côté and coworkers found that Tph2 was expressed in the neurons of the myenteric plexus in the duodenum, while Tph1 transcript was located in the nonneuronal enterochromaffin cells [Côté et al, 2003], further stressing the neuronal nature of Tph2. However, Tph2 mRNA was detected by real time RT-PCR also in the rat pineal gland, although only in trace amounts along with overwhelmingly more abundant levels of Tph1 [Sugden, 2003]. Rats have also been used to study the expression of TPH in the retina, where Tph1 levels were

predominant, yet Tph2 could also be detected there by real time RT-PCR [Liang et al, 2004]. Interestingly, according to this report, both genes follow a diurnal rhythm with levels peaking at night.

While rodent Tph2 seems to be mainly neuronal, Tph1 expression is not restricted to peripheral tissue. Albeit weak, Tph1 has been detected in the mouse raphe neurons by RPA [Walther et al, 2003], by ISH with specific probes [Gundlah et al, 2005] and by RT-PCR [Kim et al, 2005; Nakamura et al, 2006]. Similarly, it has been detected in rat brainstem by ISH [Patel et al, 2004; Malek et al, 2005] and RT-PCR [Clark and Russo, 1997].

This pattern of expression is reproduced in macaque monkeys, where TPH2 mRNA has been detected in the raphe nuclei by ISH and RT-PCR [Sanchez et al, 2005; Chen et al, 2006]. TPH1 message has also been detected in the brainstem, although not as intensely [Pecins-Thomsons et al, 1996].

Finally, human tissue has been subjected to ISH and RNA extraction to detect TPH2. In two consecutive studies, Zill and coworkers described the pattern of expression of TPH2 mRNA in humans as measured by real time RT-PCR. In the first such study, a result of averaging only two individuals, TPH2 message was relatively high in cortex, thalamus, hippocampus, amygdala and hypothalamus, but hardly detectable in heart, lung, liver, duodenum, adrenal gland and kidney [Zill et al, 2004c]. Further investigation of the human brain found TPH2 transcript levels in the raphe, in average, seven times higher than in cortex, cerebellum, hippocampus, amygdala or hypothalamus [Zill et al, 2005]. Independently of these results, TPH2 was detected in brain structures other than raphe such as dorsolateral PFC [De Luca et al, 2005b] or parietal cortex [Shamir et al, 2005].

Importantly, all of these studies detected TPH2 signal outside the raphe, where the serotonergic cell bodies reside, suggesting axonal transport of TPH2 mRNA. These studies appear to contrast with what has been seen in rodents, although Patel et al observed faint Tph2 signal in some non-raphe areas of the rat brain [Patel et al, 2004]. Human brain studies by ISH were restricted to dorsal and median raphe nuclei, where they showed specific, robust expression of TPH2 [Bach-Mizrachi et al, 2005].

Weak TPH1 expression was detected in human raphe nuclei by ISH [Austin and O'Donnell, 1999] or real time RT-PCR [Zill et al, 2005], as is the case in rodents. More interestingly, TPH1 was also amplified by real time RT-PCR from cortex, cerebellum, hippocampus, amygdala and hypothalamus [Zill et al, 2005], a result that might imply a role for TPH1 in the neuronal production of 5-HT.

Across the species, TPH2 mRNA is expressed predominantly in the raphe nuclei of the brainstem, and it may or may not migrate through the serotonergic axons to other brain structures. Its presence in the neurons of the myenteric plexus suggests that TPH2 could be strictly neuronal. Conversely, TPH1 can be found in the pineal gland, retina, mast cells, skin, enterochromaffin cells and also, importantly, in serotonergic neurons.

All in all, this pattern of expression suggests a strict control of TPH2 transcription that is activated only in a set of very particular conditions. TPH2 expression probably requires a cell committed to be neuronal and the presence of transcriptional factors that define the serotonergic phenotype. It also hints towards a relevant role of TPH2 isoform in the synthesis of neuronal 5-HT.

1.4 Relevance of TPH2

Although in minute amounts, tryptophan hydroxylase-1 is expressed in the CNS and presents higher intrinsic enzymatic activity than tryptophan hydroxylase-2. Therefore, it can be thought that TPH1, and not the newly discovered gene, controls 5-HT synthesis in the brain. For this reason, it is necessary to confirm experimentally that TPH2 is relevant.

A number of experimental approaches support the concept that TPH2 plays a role in the function of the serotonergic system *in vivo*, and consequently, that it affects 5-HT-modulated behavior, including changing the susceptibility to mental disease. Gathering evidence in cell lines and animal models indicate that TPH2 is the main enzyme responsible for the biosynthesis of serotonin in the brain, and that its activity can effectively alter 5-HT levels in diverse areas of the brain, as well as 5-HT-modulated behavioral responses. What is more, genetic association studies have started to find that TPH2 variants associate with a variety of mental disorders in which 5-HT levels are suspected to be dys-regulated. Making use of a different approach, genome-wide linkage studies are pointing to the TPH2 genomic location as a candidate locus for susceptibility to various diseases. Finally, analyses of diseased human brains suggest alterations in TPH2 expression.

1.4.1 *In vivo* function and activity of TPH2

The predicted tryptophan hydroxylation function of TPH2 has been confirmed experimentally *in vivo*. When transiently transfected with an eukaryotic

expression vector containing mouse Tph2 cDNA, COS-7 cells acquired the ability to synthesize 5-HTP [Walther et al, 2003a; Nakamura et al, 2005]. Additionally, Walther et al detected the protein in the transfected cells with commercial TPH antibodies. Similarly, pheochromocytoma 12 (PC12) cells acquired the ability to produce 5-HT (since these cells already express AAAD enzyme) after being stably transfected with hemagglutinin-tagged mouse Tph2 [Zhang et al, 2004] or human TPH2 cDNA [Zhang et al, 2005a].

Zhang et al presented the first confirmation that TPH2 controls brain 5-HT synthesis. Two variants of mouse Tph2 were found, differing in a single nucleotide in position 1473 that introduced a non synonymous change in the amino acid sequence. While one variant presented a C, coding for a Pro in position 447 (mTph2P), the other presented a G that translated into Arg (mTph2R) [Zhang et al, 2004]. When studied *in vitro*, mTph2R showed only 50% activity and an increased susceptibility to dopamine inhibition [Sakowski et al, 2006a]. Similarly, PC12 cells transfected with mTph2R produced 55% less 5-HT than the cells transfected with the mTph2P variant. These variants were found to occur naturally in different mouse strains. Strain 129X1/SvJ, expressing the more active mTph2P allele had around twice as much 5-HT and 5-HTP in frontal cortex and striatum than the BALB/cJ strain, which expresses mTph2R. The same holds true for C57BL/6 mice, which produce 45% more 5-HTP than the DBA/2 strain. These results put together led to think that Tph2 activity controls 5-HT level in different areas of the brain [Zhang et al, 2004].

Other studies confirmed these results and went further to test differences in serotonin-mediated behavior among strains. Mouse strains have been known to

respond differently in various behavioral characteristics, including aggressiveness, locomotor activity, anxiety and response to antidepressants [Wahlsten et al, 2003]. For instance, response to citalopram in the forced swimming test, an animal model of depression, was correlated with the Tph2 genotype [Cervo et al, 2005]. Two strains carrying the active form of Tph2 (C57BL/6J and129/Sv) responded to citalopram by decreasing the immobility time, whereas two other strains with the low activity isoform (DBA/2J and BALB/c) did not. To further support the role of the Tph2 polymorphism, it was shown that there was no effect of strain on citalopram brain levels, or DOPA production, and that overall locomotor activity did not play a role either. What is more, when Tph2 activity was increased in DBA/2J and BALB/c by adding tryptophan, these strains became responsive to citalopram. Likewise, when Tph2 activity was blocked in strains C57BL/6J and129/Sv using pCPA, they became unresponsive. On the other hand, response to citalopram as measured by the tail suspension test in four strains did not correlate with Tph2 genotype [Crowley et al, 2005].

The Pro447Arg polymorphism was also studied in intermale aggressive behaviour. Aggression has a well established genetic component and has been linked to the serotonergic system [Popova, 2006]. In this case, ten inbred strains were genotyped and tested for Tph activity, level of aggressiveness (percentage of mice exhibiting attack) and intensity of aggression (number of attacks). Tryptophan hydroxylating activity from brain homogenates correlated with genotype in all ten strains (C57BL/6J, CBA/Lac, AKR/J, PT/Y, C3H/HeJ, DD/He, YT/Y, BALB/cJLac, CC57BR/Mv and A/He). Tph2 was also linked to the intensity of aggression, since strains of the 447Arg genotype made in average two times less attacks to an alien

male than the high 5-HT level strains. Nevertheless, the level of aggressiveness was not correlated to genotype [Kulikov et al, 2005]. It is supposed that the two patterns of aggressive behavior respond independently to genetic mechanisms [Popova, 2006], hypothesizing that the latency before an attack is associated to choice impulsivity rather than aggressiveness. Impulsivity is also known to have a genetic component and to be related to serotonergic function [Evenden, 1999]. For this reason, a delayed reinforcement task was performed in four mouse strains (BALB/c, C57BL/6J, CBA/Ca and 129/SvHsd) to be correlated with their Tph2 genotype. Genotype was correlated with 5-HT extracellular levels in ventral striatum and medial prefrontal cortex, yet it did not correlate with impulsive behavior as measured by this test [Isles et al, 2005]. The apparent discrepancy can be explained since the delayed reinforcement task is useful to investigate choice impulsivity (related to decision-making), but not to study impulsive action (i.e. the inability to withhold a motor response), which can be tested for instance by means of a stop-task [Evenden, 1999].

All in all, it is confirmed that Tph2 exhibits tryptophan hydroxylation activity *in vivo* and that it controls 5-HT levels in the brain. A growing body of evidence also suggests that Tph2 has a direct influence on 5-HT-related behavior, since in some tests mouse strains can be divided in two groups that correlate with Tph2 genotype. In spite of the compelling evidence, it remains uncertain to assign inter-strain differences in behavior to a single SNP, albeit functional. Whether this is the case or not could be answered using mice that differed only in the Pro447Arg polymorphism, which can be created by backcross breeding [Zhang et al, 2005b].

1.4.2 Genetic association studies

Genetic association studies test the existence of a correlation between gene variants and phenotypic characteristic. A positive correlation supports the involvement of a gene in the pathogenesis of a particular disease. Most serotonergic genes have been studied for association to various diseases, with varying results. As a major candidate gene to contribute to the etiology of mood disorders, tryptophan hydroxylase-2 has been subjected to association studies since its discovery.

In the first report of any such study, Zill et al found a positive association between distinct haplotypes in the TPH2 gene and MDD. In total, they investigated ten SNPs between exons 5 and 7 in a population of German patients and controls, to found that intronic SNP rs1386494 and two haplotypes were associated to MDD [Zill et al, 2004a]. In a second report, Zill et al found the same SNP and haplotypes to be significantly correlated with suicide completion [Zill et al, 2004b]. TPH2 was also associated to MDD and suicidal behavior (not completion) in a second, more comprehensive study, in which 1798 subjects representing four populations were genotyped. In this study, fifteen SNPs spanning the entire gene were used to determine haplotypes blocks. Among other results, one haplotype ranging from intron 5 to intron 8, thought to be equal to one of Zill's associated haplotypes, was linked to depression and suicidal behavior in white American, African American and white Finnish populations. Interestingly, this same haplotype was associated to lower 5-HIAA levels in the CSF (an index of 5-HT turnover) [Zhou et al, 2005a]. Taken together, these three studies point to the existence of a functional locus

somewhere within TPH2, although possibly with modest effect on vulnerability to MDD and suicide.

The most interesting study to associate TPH2 and MDD so far reported a functional SNP present in the coding sequence. Zhang et al discovered a missense mutation in exon 11 that replaces a highly conserved Arg in position 441 for a His, causing an 80% decrease in enzymatic activity [Zhang et al, 2005a]. In all, 219 control subjects, 87 depressed patients and 60 BP patients were genotyped and the mutation was found in 9 MDD patients, 3 controls but none BP patient, thus establishing a link between the TPH2 mutation and MDD. Intriguingly, this SNP was absent in nearly 5000 subjects analyzed by several groups around the world [correspondence to Neuron, vol 48, 2005; Coon et al, 2005; Mössner et al, 2005; Bicalho et al, 2006; Delorme et al, 2006; Mössner et al, 2006], suggesting that the population genotyped by Zhang et al shared special characteristics other than clinical depression, possibly severe depression and resistance to conventional medication. Previously, other polymorphisms in TPH2 had been screened for a role in the response to fluoxetine by depressed patients. In this case, carriers of the less frequent allele in three SNPs were found more likely to respond to fluoxetine [Peters et al, 2004].

The TPH2 gene was also studied for association with bipolar disorder. A study of five intronic SNPs distributed between exons 7 and 9 only showed a weak association between a haplotype and BP [Harvey et al, 2004]. Similarly, another intronic haplotype showed a weak association to BP and suicide attempt in BP [Lopez et al, 2006]. After its discovery, studies focused on the Arg441His SNP described above, which was not found in several cohorts of BP patients [Zhang et

al, 2005a; Delorme et al, 2006]. De Luca et al have focused on investigating links between TPH2 and suicidal behavior in BP and schizophrenia. So far, results have been negative, which could be in part explained due to the moderate statistical power attained with their small sample number. In their first study, three intronic SNPs failed to associate with suicidal behavior in BP patients [De Luca et al, 2004]. As well, other three polymorphisms, two located in the 5' flanking region and the other in intron 1, were not related to suicidal behavior in BP or schizophrenia patients [De Luca et al, 2005a; De Luca et al, 2006]. Finally, no allele of the promoter polymorphisms could be correlated with TPH2 mRNA levels present in the brain of BP, schizophrenia or control subjects [De Luca et al, 2005b].

Mössner et al investigated two SNPs in linkage disequilibrium (LD), one in the 5' flanking region (G-703T) and the other one in intron 2. The two resulted associated to OCD in a transmission disequilibrium study involving 71 independent families [Mössner et al, 2005]. These two polymorphisms were not associated to panic disorder or agoraphobia [Mössner et al, 2006].

Autism was moderately linked to TPH2 in a comprehensive study that sequenced the eleven exons and both untranslated regions in 88 patients and 95 controls. Out of 18 SNPs, two (in intron 1 and 4) were significantly associated to autism [Coon et al, 2005].

TPH2 has been associated to attention-deficit hyperactivity disorder in two independent studies. In a transmission disequilibrium test analysis, Sheenan et al found one allele of an intronic SNP to be overtransmitted to ADHD children in a population of 179 Irish families. However, seven other intronic SNP included in the test failed to correlate [Sheenan et al, 2005]. In the other article, two variants in the

5' flanking region and one in intron 2 were studied in 103 German families, also for transmission disequilibrium. While the two SNPs in the regulatory region were associated to ADHD, the intronic SNP was not [Walitza et al, 2005]. Impulsivity, a behavioral trait related to ADHD, and thought to occur due to low 5-HT levels, has also been associated to TPH2, although moderately. In this case, impulsive action was measured in a response inhibition test and associated to a SNP in intron 8 [Stoltenberg et al, 2005].

Finally, a TPH2 variant has also been linked to amygdala reactivity, an index of the magnitude of emotional responses. In two independent studies, carriers of the less frequent T allele of the G-703T polymorphism showed increased amygdala activation, as measured by functional magnetic resonance imaging (fMRI) [Brown et al, 2005; Canli et al, 2005], implying a positive role of the T allele in TPH2 expression. Interestingly, amygdala reactivity was also enhanced in carriers of the short allele in the 5-HTT promoter region, the other gene that directly determines 5-HT synaptic availability [Hariri et al, 2002]. Recently, this two effects were found to be additive [Herrmann et al, 2006], further suggesting that the T allele enhances TPH2 expression.

To sum up, various association studies add to the relevance of the new gene. TPH2 has been linked at least twice to MDD, suicidal behavior and ADHD, while other positive associations, such as to autism and OCD, have been seen only once. In any case, given the high rate of false positive results in this kind of studies, more populations should be analyzed for any of the disorders. In addition,

studies could profit better ascertainment methods, more phenotype characterization and larger sample size.

The ultimate goal of finding functional polymorphisms that would tie TPH2 to a phenotype has not been achieved, with one disputed exception. Nevertheless, insights to mechanisms of disease and role of some alleles come from several TPH2 variants that have been associated to mediating phenotypes, such as 5-HIAA level in CSF, response inhibition and amygdala reactivity.

1.4.3 Genome-wide linkage studies

Genome-wide linkage studies test the existence of a correlation between a phenotype, usually a particular disease or trait, and the parental transmission of a genetic marker located in a known chromosomal locus. As opposed to association studies, linkage studies do not make any assumptions about mechanism-based candidate genes. Thus these studies might have pointed to the TPH2 locus (12q21.1) before its discovery. For example, the arm 12q has been related to depressive phenotype for some time. A predisposition locus for MDD in males was found in locus 12q22-12q23.1 [Abkevich et al, 2003], supporting the finding that neuroticism personality trait is linked to 12q23.1 [reviewed in Lesch, 2004]. A recent study further confirms a functional locus in 12q by linking 12q23.3 to depression [McGuffin et al, 2005]. Interestingly, MDD was also found to be linked to a marker 121 kb close to the CREB1 gene, a reminder that regulatory factors play equally important roles in the etiology of disease [reviewed in Levinson, 2005].

Susceptibility to bipolar disorder has been linked several times to 12q too [Craddock and Jones, 2001], while the studies beginning to scan the genome for loci predisposing to OCD [Shugart et al, 2006] or ADHD [reviewed in Heiser et al,2004] have not found any linkage to this chromosome yet.

Overall, the region in 12q near the TPH2 locus is being implicated in susceptibility to depressive phenotype and BP, but not to other mental disorders. However, many of the reports may lack power to detect genes with minor effects, resulting in false negatives. Specially when searching loci that might have weak effects on pathogenesis, such as those contributing to complex diseases, the power of genome-wide studies depends heavily on locus heterogeneity, phenotype definition and sample size [Schulze and McMahon, 2003]. For this reason, it is still too early to discard a role of TPH2 in disorders that have not been linked to 12q.

1.4.4 Changes in TPH2 expression or activity in the diseased brain

The importance of tryptophan hydroxylase-2 is further stressed by the fact that its expression or activity levels change in the brains of mental illness patients when compared to healthy controls. Direct and indirect methods have been used to correlate TPH expression or rate of 5-HT synthesis with the disease state.

To begin with, TPH2 mRNA has been measured by quantitative RT-PCR in brain homogenates from patients and controls. De Luca et al investigated three diagnostic groups of 35 individuals and found that mRNA levels in the dorsolateral PFC (BA46) of bipolar disorder patients, but not schizophrenics, were higher than controls. Interestingly, TPH2 levels correlated to lifetime use or abuse of alcohol,

but not to suicide completion, age, sex or illicit drug use [De Luca et al, 2005b]. The same technique was used to compare TPH2 expression from the parietal cortex of depressed, bipolar or schizophrenic patients to unaffected controls. In this case, there was no significant difference among the four diagnostic groups [Shamir et al, 2005].

More commonly, TPH2 expression in the disease brain has been identified by *in situ* hybridization or immunoreactivity. The majority of reports compared expression in the brainstem of depressed suicides to matched control individuals. TPH immunoreactivity (as detected with the unspecific commercial PH8 antibody) was higher in the DRN of suicides relative to controls in two studies by the same group [Underwood et al, 1999; Boldrini et al, 2005]. A third study, looking at TPH2 mRNA by *in situ* hybridization found more TPH2 message in the DRN of depressed suicide victims. It also showed that the area and volume of the DRN are equivalent in victims and controls, in agreement with Boldrini et al, suggesting that serotonergic neurons produce more TPH2 in depressed suicidal subjects. Conversely, Bonkale et al found no differences in TPH expression, using the PH8 antibody, when analyzed various subdivisions of the DRN [Bonkale et al, 2004]. The same result was obtained by Ono et al, but in this case the area studied was the prefrontal cortex (BA9), using a different antibody [Ono et al, 2002]. Additionally, TPH expression was studied in the brainstem of depressed suicide victims that suffered alcoholism. TPH immunoreactivity was 46% higher in the dorsal DRN of a group of eight alcoholics (and did not change in other subnuclei).

A different strategy to assess TPH involvement in various neuropsychiatric disorders is to measure the brain tryptophan hydroxylating activity *in vivo*. This can

be achieved by performing a positron emission tomography (PET) scan on a brain that contains α-[^{11}C]methyl-L-tryptophan, a tryptophan analog that is specifically taken up and trapped in serotonergic cells. It is proposed that the rate of irreversible trapping is proportional to the 5-HT synthesis rate [Diksic and Young, 2001]. For example, this method was used to show that 5-HT synthesis rate is decreased in the anterior cingulate cortex of medication-free depressed patients relatives [Rosa-Neto et al, 2004] or suicide attempters [Leyton et al, 2006] to their matched controls. Interestingly, lower 5-HT biosynthesis was also found in a group of medication-free subjects with borderline personality disorder [Leyton et al, 2001], which is characterized by impulsivity and aggressive behavior among other serotonin-related features.

In most of these studies, it is difficult to ascertain whether the alteration in TPH2 represents the cause of the pathology or an adaptation, either to disease or to the medication. Regardless of its origin, variation from the normal state has confirmed the involvement of TPH in disease. This yet unknown but certain role in the pathology of neuropsychiatric disorders adds to the growing genetic evidence of its function *in vivo* and provides support for further studies of TPH2, in particular the investigation of the mechanisms that regulate its levels of expression and activity.

1.5 Transcriptional regulation of gene expression

Gene expression is a multi-step process and thus its control can be exerted at many levels. Transcription is the first step in the process and the main target of regulatory mechanisms. Other controlled stages of the gene expression process include post-transcriptional modifications of the primary RNA transcript that alter its stability, transport from the nucleus to the cytosol and translation. While in many cases rapid changes in protein output following extracellular signals are controlled post-transcriptionally, tissue-specific expression of proteins and long term changes in expression are usually regulated at the transcriptional level [Latchman, 2005].

1.5.1 Transcription

Transcription, the production of an RNA molecule based on a DNA template, requires the assembly of a complex machinery that includes an RNA polymerase enzyme and a number of other auxiliary protein factors that bind the DNA and the polymerase. In the eukaryotic cell, there exist multiple isoforms of RNA polymerases that have distinct functions. RNA polymerase I and III transcribe ribosomal and transfer RNAs, whereas RNA polymerase II transcribes protein-encoding genes and some small RNAs. According to this, tryptophan hydroxylase-2 falls in the category of genes transcribed by RNA polymerase II.

Regardless of the polymerase involved, the formation of the initiation complex is conceptually similar. The promoter, a DNA region around the transcription start site (TSS), recruits transcription factors that establish protein-

protein interaction with the RNA polymerase and several other proteins that do not bind DNA. Some of these transcription factors bind to the core promoter and are necessary for initiation in almost every gene (general transcription factors, GTF). On the other hand, other factors can interact only with certain promoters, or under particular conditions, to activate or repress transcription.

Core promoters of genes transcribed by RNA polymerase II are composed of various DNA elements that bind GTFs and allow the formation of the initiation complex. The binding sites that are present vary from gene to gene, as does the sequence of each particular element. However, some features are shared by a large number of promoters. To begin with, an AT-rich element, the TATA box, is found in numerous, but not all, genes around 25 to 30 bp upstream of the TSS. The TATA box binds the TATA-box binding protein (TBP), which recruits up to fourteen TBP-associated factors (TAF). This multi-protein assembly is the transcription factor IID (TFIID), on top of which the initiation complex is built by the binding of other GTFs, such as TFIIE, -F and –H, and finally the polymerase enzyme [reviewed in Butler and Kadonaga, 2002]. In addition, some of the promoters that present a TATA box also possess immediately upstream a CG-rich sequence called BRE, for TFIIB-recognizing element. More often, core promoters include an initiator sequence (Inr) that encompasses the TSS from -2 to +4. Finally, common downstream sequences have been found in many core promoters. Kadonaga et al found the downstream promoter element, a site that also binds TFIID and is most common in TATA-less promoters [reviewed in Kadonaga, 2002] and the motif 10 element (MTE), which also can act independently of the TATA box

[Lim et al, 2004]. The combination of some of these elements and others allows the formation of the initiation complex. However, this event is tightly regulated.

1.5.2 Regulation of transcription

The formation of the initiation complex and its efficiency to start transcription depend on multiple stages of control that interact to attain the adequate rate of gene expression. These stages include the presence of transcription factor binding sites (TFBS) further upstream or downstream from the core promoter that may bind factors to alter the initiation complex characteristics, or other factors that control the condensation state of the chromatin at the promoter, determining the ability of the initiation complex components to even reach the DNA [Latchman, 2005].

Core promoters of genes transcribed by RNA polymerase II are surrounded by short regulatory elements that are capable of binding transcription factors. These elements are located in clusters in the proximal promoter region, so that transcription factors can interact with each other and function synergistically [reviewed in Kadonaga, 2004]. In addition, clustering of TFBS allows the binding of a subset of factors depending on a particular condition, thus achieving a combinatorial control of gene expression [Remenyi et al, 2004]. Once bound, transcription factors can interact with the basal transcriptional complex directly or via recruitment of coactivators and corepressors. Alternatively, transcription factors can modify the chromatin structure around the promoter [reviewed in Kadonaga, 2004].

For instance, one of the transcription factors commonly found in the proximal promoter region is Sp1. Sp1 is a ubiquitously expressed protein, member of the Sp family of zinc-finger transcription factors. It binds GC boxes (consensus sequence GGGGCGGGG) to activate transcription of both housekeeping and inducible genes, although there are some instances where it acts as a repressor [reviewed in Li et al, 2004b].

Alternatively, short regulatory sequences called enhancers or silencers can be located thousands of bp away from the TSS, either upstream, downstream or within the gene being transcribed. These elements can significantly alter the basal rate of transcription, although they do not promote transcription by themselves. The mechanisms of action of these transcription factors are similar to those in the proximal promoter, but they require bending and looping of the DNA strand to act on the TSS region [Latchman, 2005].

Remodelling of chromatin structure constitutes a different aspect of transcriptional regulation. DNA strands are compacted in bead-like structures called nucleosomes, each of which is comprised of about 200 bp and eight histone proteins (two of each of H2A, H2B, H3 and H4). Furthermore, contiguous nucleosomes bend onto themselves to adopt a solenoid shape, involving histone H1 [Alberts et al, 1995]. Since this highly condensed structure prevents gene expression, transcriptionally active regions present a more open conformation, as evidenced by the enhanced sensitivity to DNAse I seen in these areas [Latchman, 2005]. Alternation between the two states is achieved through chromatin-remodelling, a process carried out by multi-protein enzymatic complexes that

covalently modify histones, like histone acetyltransferases, or that alter histone-DNA contacts, like SWI/SNF [de la Serna et al, 2006]. Covalent modification of histones, including acetylation, methylation, sumoylation, phosphorylation and ubiquitination, contribute to determine transcriptionally active and inactive regions of the DNA. In particular, histone acetyltransferases (HAT) can acetylate specific lysine residues, loosening DNA-histone interactions to favor transcription [Latchman, 2005]. Importantly, this level of regulation is not separate from the action of short regulatory sequences, since many of the transcription factors bound to them can direct the assembly of the enzymatic complexes that modify histones and attract chromatin remodeling.

Mechanisms of higher order than the local chromatin structure and short regulatory sequences are also necessary for proper gene expression. Enhancers and silencers act over long genomic distances and therefore require the looping and spatial organization of the chromatin so that the effect is transmitted to the right genes and not to others. This specificity can be achieved by insulator elements that protect genes from the influence of remote regulatory sequences not directed to them. Alternatively, dynamic chromatin boundaries may define gene expression domains separated from inactive DNA [West and Fraser, 2005]. What is more, these domains can exist as nuclear areas enriched in transcriptional machinery, or transcription factories, associated with higher transcriptional activity [Dillon, 2006]. Thus, the rate of transcription of a gene depends not only on the local state of chromatin condensation, but also in the large-scale chromatin organization.

Finally, there is recent evidence for the participation of natural non-coding RNA in the control of transcription [Goodrich and Kugel, 2006]. Non-coding RNAs have been found that target chromatin or RNA polymerase II, act as coactivators, regulate nuclear trafficking or alter the activity of a repressor, such as the neural-restrictive silencer factor / repressor element-1 silencing transcription factor (NRSF / REST).

1.5.3 Regulation of transcription by REST

Tissue-specific gene expression arises from accurately-timed developmental transcriptional programs that make use of the mechanisms described above. For instance, a complex system of activators and repressors trigger neuronal differentiation in some cells while it shuts off neuronal genes in others. This neuron-specific expression is achieved in part through the action of REST, a ubiquitous repressor that prevents expression of neuron-restricted genes in non neuronal cells as well as their premature transcription in early neuronal precursors.

REST was initially discovered as a zinc finger transcription factor that repressed expression of the NaV1.2 channel and the SCG10 genes in non neuronal cells by binding a 21-bp-long site, the repressor element-1 (RE-1) [reviewed in Jones and Meech, 1999]. Subsequently, sequences similar to RE-1 were found in more than a thousand gene promoters [Zhang et al, 2006], and validated in more than thirty, including tyrosine hydroxylase [Kim et al, 2006], the cholinergic gene locus [Hersh and Shimojo, 2003], brain-derived neurotrophic factor (BDNF) [Timmusk et al, 1999] and the 5-HT1A receptor [Lemonde et al,

2004]. In all cases, as expected, REST represses non neuronal transcription. In agreement with this concept, REST was found to be expressed in non neuronal tissues and neural progenitors, but rarely in post-mitotic neurons. Furthermore, REST KO mice showed evidence of premature neuronal differentiation [Chen et al, 1998].

REST repressing activity depends on two domains that interact with distinct sets of corepressors (mSin3, N-CoR, Co-REST and others). While mSin3 binds the N-terminal domain and recruits histone deacetylase-1 and -2 (HDAC1 and HDAC2), Co-REST binds the C-terminal domain to bind SWI/SNF, a multi-protein complex for chromatin remodeling [Lunyak et al, 2004; de la Serna et al, 2006]. This flexibility allows REST to have a broader function, including regulation of neuronal differentiation, activation of transcription in mature neurons and even tumor-suppressing and oncogenic actions [Majumder, 2006].

Given that TPH2 presents a neuron-restricted pattern of expression, it is possible that REST participates in its transcriptional regulation. If this was the case, it would be expected that the TPH2 promoter bear a RE-1 sequence that repressed activity in early neuronal stages and non neuronal cell types. Conversely, in neurons, the TPH2 promoter would be subjected to the regulation of constitutive and activity-dependent transcription factors.

1.5.4 Regulation of transcription by synaptic activity

Synaptic activity often results in post-translational modifications with immediate consequences in neurotransmission, yet it may also induce long-term

adaptations of the neuron by altering gene expression, particularly at the level of transcription [Kandel, 2001]. Thus neurons possess the ability to develop long-lasting adaptations to transient inputs, such as action potentials. Although neurotransmitters can contribute to these changes in gene expression through various signaling pathways, Ca^{++} is the main second messenger linking synaptic activity to transcription [West et al, 2001].

Synaptic activity implies in all cases the elevation of intracellular Ca^{++} concentration. However, heterogeneity in the firing frequency, burst duration, and overall pattern of action potential firing lead to diversity in the magnitude, duration and subcellular localization of the Ca^{++} peaks. Differences in Ca^{++} transients, related to distinct routes of entry, result in activation of different signaling cascades and therefore activation of a particular transcriptional program [Fields et al, 2005]. In summary, the neuron can develop a stimuli-specific adaptive response.

In a synaptic event, extracellular Ca^{++} enters the cytoplasm through voltage-gated Ca^{++} channels (VGCC) or glutamate receptors, such as the N-methyl-D-aspartate (NMDA) receptor or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor. Alternatively, elevation of cytosolic Ca^{++} can result from opening of internal stores like the endoplasmic reticulum (ER) [West et al, 2001].

VGCCs are multi-subunit transmembrane proteins that open upon depolarization, to inactivate later due to repolarization or via a Ca^{++}-induced mechanism [Catterall, 2000]. L-type VGCCs, a subgroup of channels that inactivate slowly, are especially related to transcriptional regulation even when

Ca^{++} enters the cell mainly through other gates [West et al, 2001]. L-type channels are located in dendrites and cell bodies and coupled to Ca^{++} / calmodulin (Ca^{++} / CaM). This coupling is necessary to transduce the signal to the nucleus via activation of Ca^{++} / calmodulin-dependent protein kinases (CaMK) or Ras-MAPK (mitogen-activated protein kinase) pathways. The activation of these cascades translate into the phosphorylaton of transcription factors such as cAMP-responsive element binding protein (CREB), myocyte-enhancer factor (MEF) or the nuclear factor of activated T cells (NFAT) and into the transcription of immediate-early genes [reviewed in West et al, 2002].

In addition to VGCCs, Ca^{++} enters the cell through glutamate NMDA receptors, which require simultaneous binding of glutamate and membrane depolarization to be fully active. Opening of these channels is also linked to changes in transcription, such as immediate-early gene activation [West et al, 2002]. Like L-type channels, synaptic NMDA receptors have been shown to couple to the Ras-MAPK pathway and phosphorylate CREB [Hardinghan et al, 2002].

Ca^{++}-induced Ca^{++} release (CICR) from the endoplasmic reticulum (ER) and the nuclear envelope can contribute to synaptic activity-dependent transcription. Activation of both 1,4,5-inositol triphosphate receptors (IP_3R) and ryanodine receptors (RyR) by synaptic activity opens the internal stores and elevates intracellular Ca^{++} concentration [reviewed by Fields et al, 2005].

Importantly, Ca^{++} entry usually occurs through multiple routes simultaneously and therefore the pathways overlap and cross-talk. In the end, the neuron integrates the information from the various signaling pathways to activate one or another set of transcription factors.

The last step in activity-dependent regulation of transcription is the alteration of transcription factor activity. Most of these factors are the target of protein kinases or phosphorylases, although there are some solely regulated by direct Ca^{++} binding, such as the downstream regulatory element-antagonist modulator (DREAM) [Carrion et al, 1999]. In addition to CREB, examples of transcription factors regulated by activity-dependent phosphorylation in neurons include the Ca^{++}-responsive transactivator (CREST) [Aizawa et al, 2004], neurogenic differentiation-2 (NeuroD2) [Aizawa et al, 2004] and 5' repressor element under dual repression binding protein-1 (Freud-1) [Ou et al, 2003].

CREB belongs to a family of bZIP transcription factors, also referred to as CREB. Beside various CREB isoforms, this family includes CREB-modulator (CREM) and the activating transcription factor-1 (ATF-1) [Lonze and Ginty, 2002]. These related factors can assemble in homo- or heterodimers to bind the cAMP-responsive element (CRE, consensus sequence TGACGTCA). Functional CRE sites have been found in the proximal promoter of numerous genes, including immediate-early gene c-Fos, BDNF, TH and CREM. In agreement with the long list of target genes, CREB has been associated to learning and memory, mood disorders, addiction, development of the CNS, circadian rhythm and a number of functions not related to the nervous system [Lonze and Ginty, 2002; Carlezon et al, 2005].

While there is evidence of phosphorylation-independent activity [Blendy, 2006], CREB action is primarily observed after phosphorylation at Ser133, a residue located in the kinase inducible domain (KID) [West et al, 2002].

Phosphorylation of Ser133 allows CREB to recruit the CREB-binding protein (CBP), a transcriptional coactivator critical to CREB activating function [West et al, 2002]. CBP acts by stabilizing GTFs in the initiation complex and also by modifying chromatin structure with its HAT activity [Lonze and Ginty, 2002].

Ser133 phosphorylation is the common result of a number of diverse stimuli. For instance, an increase in cAMP, such as that resulting from $G_{\alpha s}$ protein stimulation, activates protein kinase A (PKA), which in turn phosphorylates Ser133. Similarly, neuronal activity and the subsequent increase in Ca^{++} lead to Ser133 phosphorylation, this time by CaMK IV or ribosomal S6 kinases (RSK) [West et al, 2002]. Nevertheless, signals differ from each other in their kinetics, the half-life of the phosphoCREB (pCREB) isoform, the simultaneous activation of phosphatases or the phosphorylation of alternative residues, like Ser142 and Ser143. Thus, CREB action becomes very sensitive to the source of the signal, allowing the neuron to modulate gene expression as it receives external information.

Regulation of transcription is a crucial regulatory step to achieve desired gene expression levels, either in basal state or to adapt to varying extracellular conditions. In neurons, these varying conditions often implicate synaptic activity, which translates into transcriptional adaptations through a variety of Ca^{++}-related signaling pathways. These mechanisms target numerous neuronal genes, including tyrosine hydroxylase (a gene with a function analogous to that of TPH2) and hence might also be relevant for TPH2.

1.6 Transcriptional regulation of TPH2 and related cases

The regulation of TPH mRNA and protein expression has been extensively studied. However, due to the recent discovery of TPH2, the majority of the previous studies do not distinguish between the two isoforms. At the time, available antibodies recognized both isoforms, while *in situ* hybridization studies lacked proper specificity controls. Thus, these studies provide only a measure of total TPH protein or RNA expression. More recently, a number of reports have begun to investigate TPH2 specifically. While only one report addressed the molecular mechanisms of transcriptional regulation acting on the promoter, most others investigated the modulation of TPH2 expression by cues that are known to alter central serotonergic function, such as sexual hormones or the circadian rhythm.

As opposed to the TPH2 promoter, the regulatory region of human TPH1 gene is well characterized. Data on how this promoter responds to certain signals may contribute to understand the results from TPH expression studies that did not distinguish between isoforms. Similarly, further information about TPH2 can be inferred from analysis of tyrosine hydroxylase transcriptional regulation, a gene with a physiologically homologous function and hence likely similar regulation.

Data provided by TPH expression experiments, together with TPH1 and TH regulatory information, can contribute to devise hypothesis about the pathways that converge on the TPH2 promoter.

1.6.1 Regulation of TPH2 expression

The only study specifically addressing transcription by the TPH2 promoter reported the finding of a double RE-1-like sequence around position -113. Notably, this element is conserved in the human, chimpanzee, mouse, rat and rabbit promoters. When cloned before a reporter-promoter and transfected into C6 glioma cells, this element strongly repressed transcription [Mueller and Patel, 2005].

Expression of Tph2 mRNA, as well as total TPH protein and activity levels follow a circadian rhythm in the rat dorsal and median raphe nuclei, the serotonergic areas projecting to the circadian system [Malek et al, 2005]. Interestingly, Tph2 mRNA levels exhibited the same pattern under light-dark cycle conditions or constant darkness [Malek et al, 2005]. These properties are coincident with those of Tph1 in the pineal gland [Bernard et al, 1999], where the minute levels of Tph2 mRNA are also circadian [Sudgen, 2003]. Taken together, these results might indicate a shared regulatory feature. Since expression of clock genes is not detectable in the raphe [Malek et al, 2005], it is possible that one of these shared features is cAMP or Ca^{++} dependency necessary to follow a circadian rhythm.

Some mental illnesses, notably depression, and other serotonin-related disorders are characterized by gender-specific features, suggesting that sexual hormones may regulate serotonin function. For this reason, estrogen is one of the most studied putative modulators of TPH2 expression. Work by the Bethea group has established a model of surgical menopause in macaque monkeys and shown

that estrogen (E) elevates TPH mRNA and protein levels in the dorsal raphe [Pecins-Thompson et al, 1996; Bethea et al, 2000]. Later, this result was confirmed for TPH2 by specific ISH and RT-PCR [Sanchez et al, 2005]. While macaque serotonergic neurons express estrogen receptor-β (ERβ) [Gundlah et al, 2001], no estrogen-responsive elements were found in the macaque TPH2 promoter. Similar results showed that E increased TPH2 mRNA in certain subregions of the rat MRN and DRN [Hiori et al, 2006]. On the other hand, one article reported no changes in mouse raphe TPH2 mRNA after E treatment [Clark et al 2005]. All in all, estrogen appears to modulate TPH2 expression, although it is unclear whether ERβ acts directly on the TPH2 promoter or via indirect mechanisms.

1.6.2 Transcriptional regulation of TPH1

Although TPH1 and TPH2 genes differ in their patterns of expression and physiological role, the transcriptional regulation of TPH1 is relevant to the study of TPH2 in order to gain insights to possibly shared regulatory pathways, such as those leading to circadian rhythm expression. Moreover, knowledge of TPH1 regulation may contribute to understand the significance of expression data from unspecific TPH protein studies.

The human TPH1 gene is transcribed from only one promoter, in spite of the multiple 5'-UTR splicing variants reported [Boularand et al, 1995a]. A 2-kb-long fragment from the 5' flanking region was shown to be transcriptionally active in

Tph1-positive primary pinealocytes as well as in Tph1-negative PC12 cells [Boularand et al, 1995b]. Transcriptional activity of deletion constructs was induced by cAMP, probably through an inverted CCAAT box [Boularand et al, 1995b]. In pinealocytes, deletion constructs carrying the inverted CCAAT box recruited nuclear factor-Y (NF-Y) to confer cAMP-inducibility by a CRE-independent mechanism [Cote et al, 2002]. The response to cAMP stimulation, as well as to Ca^{++}, has been linked to the circadian expression of Tph1 in the pineal gland of rat [Ehret et al, 1991] and chick [Florez and Takahashi, 1996].

Regarding the tissue-specificity of TPH1 expression, it was observed that the promoter deletion constructs worked equally well in pinealocytes and PC12 cells, so these studies could not identify elements that conferred tissue-specificity. However, studies of the murine Tph1 promoter localized pineal-specific elements approximately 5.8 kb upstream of the TSS [Yim et al, 2000].

BDNF contributes to the growth of serotonergic neurons and their differentiation into a fully developed serotonergic phenotype [Siuciak et al, 1996; Galter and Uniscker, 2000b]. For this reason, TPH protein and Tph1 mRNA expression were studied after BDNF treatment in several systems. To begin with, BDNF increase total TPH protein in RN46A cells, a cell-line model of serotonergic neurons [Eaton et al, 1995], although the same treatment could not increase transcription of a luciferase reporter gene fused to the Tph1 promoter [Siuciak et al, 1998]. In addition, BDNF increased Tph1 mRNA in the mouse DRN, RN46A cells [Siuciak et al, 1998] and rat raphe primary neurons [Galter and Unsicker, 2000a].

These studies used RT-PCR followed by sequence confirmation, so it can be assumed that they were specific for Tph1. In agreement with recent specific reports of Tph1 expression in the raphe, all three cases (mouse DRN, RN46A cells and rat raphe cultures) reported minute amounts of mRNA that were detected with difficulty. Since changes in these small quantities correlated with TPH protein expression, now known to be overwhelmingly derived from Tph2, it can be speculated that BDNF may act on the Tph2 gene as well. Importantly, BDNF stimulation activates numerous cascades in CNS neurons, but notably it activates activating protein-1 (AP-1), CREB and causes elevation of cytosolic Ca^{++} concentration [Gaiddon et al, 1996; Rose et al, 2004].

Similarly, rat raphe Tph1 mRNA, expressed in very low amounts, increased in response to immobilization stress, as measured by RT-PCR [Chamas et al, 1999]. This increase was correlated with raphe Tph protein elevation [Chamas et al, 2004] and Tph activity in different brain areas, again suggesting that stress may also affect Tph2 expression.

1.6.3 Transcriptional regulation of TH

TH is the rate-limiting enzyme in the synthesis of catecholamines and thus an enzyme that shares some functional characteristics with TPH2. In addition to sequence homology, it is possible that both genes are subjected to similar regulatory mechanisms. Therefore, study of TH transcriptional regulation may provide some insights into TPH2 regulation.

TH is expressed in catecholaminergic neurons and in chromaffin cells of the adrenal medulla, both cell types of neural origin. To study the genomic elements required for this specificity, transgenic mice were prepared carrying a reporter gene linked to the 5' flanking region of the TH gene. The shortest sequence to convey tissue-specificity with minimal ectopic expression was 4.5 kb long, from the rat sequence, which includes consensus RE-1 sites [Schimmel et al, 1999]. Transgenic gene expression by the human 5' region has not provided conclusive results, but three functional RE-1 sites have been located in the human TH promoter that conferred certain specificity when transfected into cell lines [Kim et al, 2003; Kim et al, 2006].

Spatial and temporal regulation of TH gene expression is subjected to an intricate combination of transcription factors acting on its 5' flanking region. In the first 250 bp upstream of the TSS, the TH promoter concentrates the TATA box, two CRE sites, an AP-1 element and others [Kumer and Vrana, 1996; Sabban and Kvetnansky, 2001]. A CRE site at position -40 and the AP-1 site at -200 are responsible for the Ca^{++}-induced activation of TH transcription observed after depolarization [Nagamoto-Combs et al, 1997]. Furthermore, combination of these sites with others integrates transcriptional regulation by multiple signals, such as nerve growth factor (NGF), hypoxia and glucocorticoids [Kumer and Vrana, 1996]. Interestingly, in coincidence with the response observed for TPH, TH mRNA levels were modulated by estrogen treatment [Maharjan et al, 2005] or various stress-inducing protocols [reviewed in Sabban and Kvetnansky, 2001].

Preliminary information on the regulation of TPH2 expression indicates that multiple pathways converge to the TPH2 promoter, as is the case for the TH gene. Stress, estrogen, BDNF and others have been shown to alter total TPH protein expression or TPH2 mRNA in the raphe neurons, but the mechanisms involved have not been explored in detail. Although pathways are not yet known, it seems possible that neuronal activation or direct elevation of intracellular Ca^{++} mediate action of some of these stimuli. These issues as well as the detailed analysis of TPH2 transcription at a molecular level may profit from homogeneous system models that reproduce *in vitro* the functioning of a serotonergic neuron.

1.7 Cell culture models to study serotonergic genes

Cell-culture models are extremely useful to study a number of events that take place within the cell, like gene expression, signaling cascades, cell cycle progression and different aspects of metabolism. In addition, these systems allow the direct investigation of the effect of drugs, hormones and other stimuli on a given cell type, independently of their effect in neighboring cells. Finally, cell culture models represent a more homogeneous and biochemically accessible alternative to the use of animals or primary cultures. However, these models generally compromise fidelity to the source tissue in exchange for ease of use.

Models resembling serotonergic neurons are not abundant, in part due to the difficulty of establishing neuronal cell lines. Additionally, many available models only partially reproduce the complexity of the serotonergic neuron phenotype,

which includes synthesis, storage, release, active reuptake and high affinity autoreceptor binding of 5-HT. Conversely, RN46A cells and primary cultures from raphe nuclei are among the systems that more closely resemble the serotonergic neurons *in vivo*.

1.7.1 RN46A cell line

RN46A cells were derived from primary neurons extracted from the medullary raphe nucleus of E13 embryonic Sprague Dawley rats [White et al, 1994]. The neurons were infected with a retrovirus carrying the temperature-sensitive mutant of the SV40 large T antigen. The G418-resistant line RN46 was subcloned by limiting dilution, yielding the RN46A cell line. RN46A cells are temperature-sensitive immortal neuronal cells that can continue their differentiation only when grown in permissive conditions. In other words, cells proliferate at 33ºC when the large T-antigen is active, but switching to non permissive temperature (39ºC) disrupts the T-antigen and cells constitutively differentiate to a serotonergic phenotype [Whittemore and White, 1993; White et al, 1994].

This strategy led to a cell line that expressed a complete serotonergic phenotype when differentiated. Undifferentiated, proliferating RN46A cells presented fibroblast-like morphology and did not express neuronal markers, whereas differentiated cells switched to a neuronal morphology, extending axons [White et al, 1994] (Figure 1.2). When differentiated, RN46A cells expressed more neurofilaments, TPH, AAAD, trk receptors, 5-HTT, VMaT and more specific 5-HT1A binding [White et al, 1994; Eaton et al, 1995], although 5-HT and TPH

protein levels have been reported to be low [Eaton et al, 1995; Bethea et al, 2003]. Treating the differentiating cells with a combination of BDNF and chronic partial depolarization increased 5-HT levels and improved the expression of serotonergic markers, including TPH [Eaton et al, 1995], in agreement with the evidence observed in serotonergic neurons *in vivo* [Siuciak et al, 1998]. In addition, RN46A cells expressed 5-HT1A autoreceptor [Kushwaha and Albert, 2005] and also 5-HT1B, yet their overlapping subcellular distribution differed from *in vivo* observations [Rumajogee et al, 2005]. RN46A cells resembled serotonergic neurons also in functional aspects, since they were shown to take up 5-HT [Eaton et al, 1995; Koldzic-Zivanovic et al, 2006], or package it in vesicles and release it upon KCl-induced depolarization [Bajali et al, 2005]. Further studies found even more similarities between the RN46A model and serotonergic neurons. For example, Bethea and coworkers characterized the protein expression of nuclear receptors in the differentiated cells, finding estrogen receptor-β (ERβ), androgen receptor and NF-κB, but not estrogen receptor-α (ERα) or progestin receptor (PR), thus completing an expression profile in absolute agreement with that of the rodent raphe 5-HT neurons [Bethea et al, 2003; Koldzic-Zivanovic et al, 2004]. When used to study the promoters of serotonergic genes, RN46A cells showed a distinct pattern of transcriptional regulation due to the presence of a specific combination of transcription factors, thought to resemble that of a pre-synaptic serotonergic neuron [Mortensen et al, 1999; Storring et al, 1999; Czesak et al, 2006].

To sum up, differentiated RN46A cells express a complete neuronal serotonergic phenotype, strikingly similar to their *in vivo* counterparts. In spite of

low TPH and 5-HT levels, these studies taken together support the use of RN46A cell line as a model system for the study of raphe serotonergic neurons.

1.7.2 Rat raphe primary cultures

Primary culture of neurons from rat midbrain is a well established method for the study of the molecular biology of serotonergic neurons. Usually, tissue from the rostral rhombencephalon along the midline is dissected out from rat embryos at days E13 to E15, after the first 5-HT-positive cells have appeared but before they send extensive projections [Héry et al, 1999; Galter and Unsicker, 2000a; Lautenschlager et al, 2000; Czesak et al, submitted]. The dissociated tissue can be cultured in conditions that stop glial proliferation, yet not necessarily that favor serotonergic differentiation, resulting in a highly heterogeneous population of neurons [Czesak et al, submitted]. In fact, the realization that only between 1 and 8% of the cells are 5-HT-positive constitutes the main drawback of this model system.

Nevertheless, the serotonergic cells can be readily detected and their functionality studied. To begin with, specific reuptake of 5-HT could be demonstrated in the cultures as early as day *in vitro* 3 (DIV 3), a mechanism that was inhibited by fluoxetine [Héry et al, 1999]. Secondly, serotonergic cells in culture expressed VMaT2 and shown exocytic 5-HT release by DIV 9 [Lautenschlager et al, 2000]. Additionally, 5-HT1A and 5-HT1B autoreceptors could downregulate 5-HT synthesis and release [Héry et al, 1999], a regulatory mechanism that is also seen *in vivo* [Barton and Hutson, 1999]. Finally, just like

RN46A cells, serotonergic neurons in culture were reported to upregulate TPH expression if treated with BDNF, a process shown to depend on functional trk B receptors [Galter and Unsicker, 2000b].

Primary cultures of rat raphe constitute an excellent model to study the molecular and cellular biology of serotonergic neurons, because these cells seem to present many of the same properties seen *in vivo*. However, two major shortcomings have to be acknowledged. Firstly, analysis by methods that make use of whole-culture extraction of protein or nucleic acid are not selectively targeting serotonergic neurons, but rather an heterogeneous population of cells. Secondly, since development most likely is not mimicked *ex vivo*, cultured neurons may not be expressing the same set of genes as their *in vivo* counterparts, and therefore lack key participants of signal transduction or gene regulation cascades.

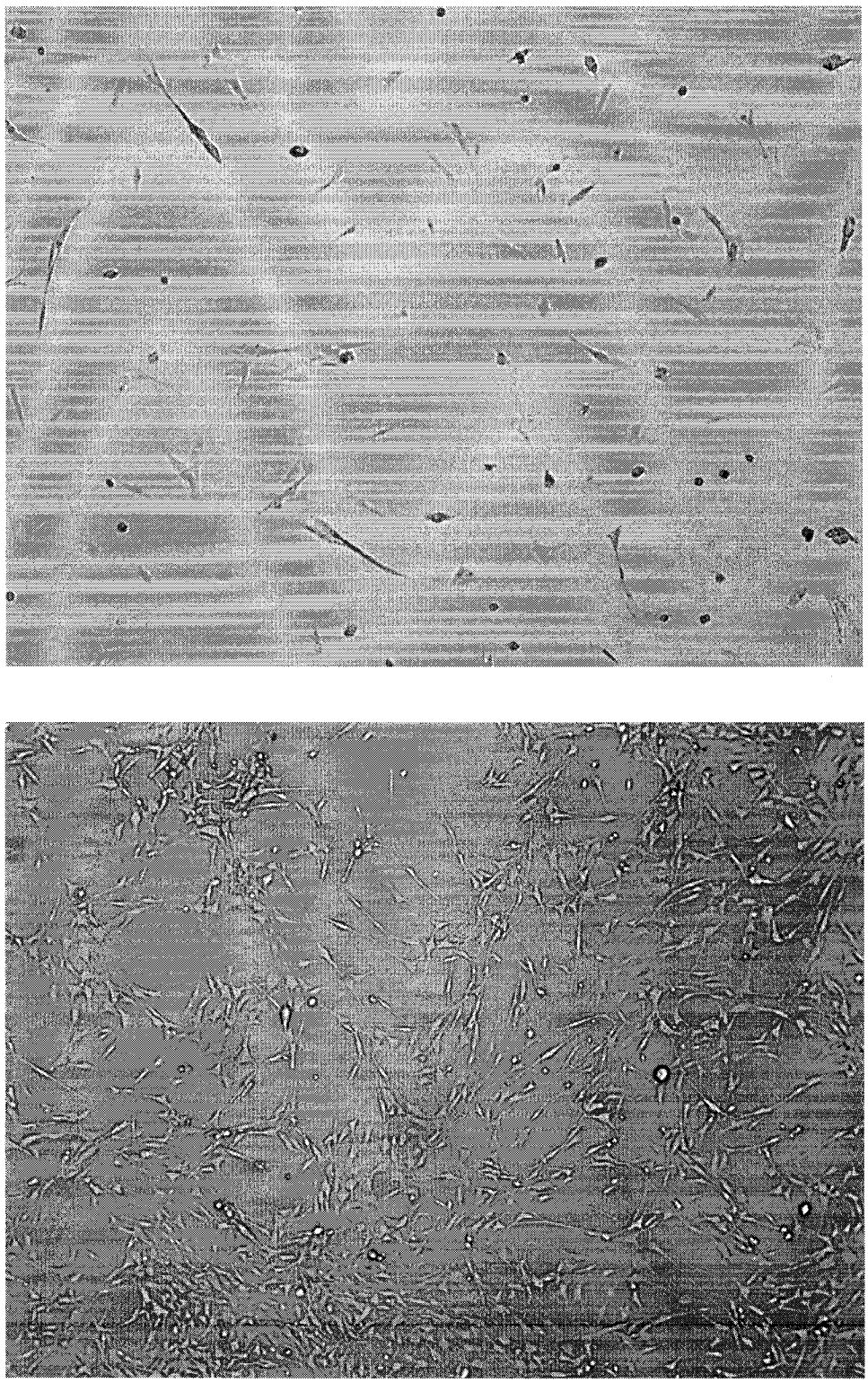

Figure 1.2 – Photomicrographs of undifferentiated (top) and differentiated (bottom) RN46A cells.

1.7.3 Other models of serotonergic cells

There exist many other cell lines that have been studied as models of serotonergic neurons. However, few exhibit a complete serotonergic phenotype, like RN46A cells do. In general, cells obtained from peripheral tissues like skin, carcinoid tumors from the gut or other sources presented some markers but were not neuronal or alternatively, did not synthesize 5-HT [reviewed in Eaton et al, 1995]. Importantly, the study of the transcriptional regulation of neuronal TPH strictly requires these two characteristics.

Recently, a promising report was published in which rhesus monkey embryonic stem cells had been differentiated to an apparently complete serotonergic phenotype, although a cell line was not yet established [Salli et al, 2004]. These neuronal cells produced 5-HT and expressed TPH1, TPH2, 5-HTT, 5-HT1A, PR, ERβ and ERα, exactly as seen in the macaque serotonergic neurons of the DRN.

Nevertheless, differentiated RN46A cells and primary cultures from the midbrain continue to be the best available systems to study expression and regulation of serotonergic genes, in particular of neuronally-restricted TPH2.

1.8 Summary of rationale to study TPH2 transcriptional regulation

The serotonergic system in the CNS modulates a plethora of physiological functions, from mere autonomic processes like endocrine activity and nociception

to more complex regulation of behavior such as appetite, emotions and cognitive abilities. Accordingly, dysregulation of serotonergic neurotransmission appears to be a contributing cause of many psychiatric and non-psychiatric pathologies. Proper serotonergic function requires adequate production of serotonin, which is controlled in part by the level and activity of the enzyme catalyzing the first and rate-limiting step in 5-HT biosynthesis, tryptophan hydroxylase. In the CNS, the most relevant TPH isoform appears to be TPH2, a gene expressed only in serotonergic neurons. Importantly, there is increasing evidence that TPH2 is linked to the mechanisms of mental illness.

Regulation of transcription is a well-known mechanism to control gene expression, especially in the long-term. Changes in the transcriptional rate can be carried over and lead to changes in the levels of an end-product, especially in the cases of genes encoding rate-limiting enzymes. In addition, many cell-types, particularly neurons, use changes in intracellular Ca^{++} concentration as a means to link extracellular events to changes in gene expression.

Taken together, it can be hypothesized that the rate of TPH2 gene transcription has an impact in serotonin brain levels, and therefore also in the functioning of the various systems modulated by 5-HT. Studying TPH2 transcription will provide insights to the functioning, and malfunctioning, of the serotonergic system, hence contributing to our understanding of mental illness pathogenesis. In particular, the hypothesis of my work are that (1) the transcription of the TPH2 gene is regulated by specific DNA elements present in its 5' region and (2) there are DNA elements in the TPH2 promoter responsible for cell-specific activity-dependent increase in TPH2 transcription.

Working under these two hypotheses, the objectives of my project are to identify and characterize the TPH2 promoter region, in terms of its functional DNA elements and the activation/repression pathways that act upon them and to determine whether TPH2 transcription is activity-dependent in neuronal cell types.

This was achieved by making use of reporter gene assays of different deletion constructs transfected into differentiated RN46A cells, rat raphe primary neurons, HEK 293 and L6 rat myoblasts. Characterization of the promoter included the study of the transcriptional response to Ca^{++} mobilization, elicited by ionomycin treatment, also by means of reporter gene assays. Furthermore, the effect of ionomycin- or KCl-induced Ca^{++} mobilization on endogenous transcription was tested by measuring Tph2 mRNA by quantitative RT-PCR.

The results obtained in this work constitute the first report and characterization of the human TPH2 promoter, as well as the first indication of the cell-type specific Ca^{++}-dependence of TPH2 transcription. Further studies should address the mechanisms by which this response is elicited, whether the increase in transcription leads to an increase in translation and finally, if this is corroborated *in vivo*, the possible involvement of defects in this pathway in the pathogenesis of 5-HT-related illness.

CHAPTER II - MATERIALS AND METHODS

2.1 Sequence analysis

DNA sequences for the human and rat 5' flanking regions of the TPH2 gene were obtained from the NCBI website [www.ncbi.nlm.nih.gov]. Sequence alignments were obtained by the ClustalW method, using MegAlign software (Lasergene) or using the Blast 2 Sequences software, available online at the NCBI website.

Transcription factor binding sites (TFBS) were detected scanning the sequence from -5073 to +142, relative to the transcription start site of the human TPH2, with the Transcription Element Search Software (TESS) [http://www.cbil.upenn.edu/tess]. A fragment from positions -5073 to +879 of the reported transcriptional start site (TSS) was processed with Promoter Scan Version 1.7 [http://thr.cit.nih.gov/molbio/proscan], which predicts promoter regions and transcription start sites based on homology to previously reported eukaryotic promoters that use RNA Polymerase II.

2.2 Plasmid construction

The TPH2 putative promoter region was cloned from human genomic DNA in two steps, and the PCR products were ligated to result in a 5 kb segment. Two

sets of primers were used: 5'-GTG ATG GCC TGT GCT TGT AGT CC-3' and 5'-AAA CCC TCT CCG TGC CCA GTA T-3' for the fragment adjacent to the gene and 5'-CCT GAG CAG CTG GGA ATA CT-3' and 5'-ACG CAC TTT ACA TGA CAG AAT ACC-3' for the more upstream part. A SpeI/BamHI fragment of the ligated product, which contained the 5'-UTR, was cloned upstream of the luciferase coding sequence in the pGL3 Basic vector (Promega). Five constructs were prepared from restriction fragments of this initial plasmid: -1402 (PleI/BamHI), -1082 (ScaI/BamHI), -683 (HindIII/BamHI), -503 (EcoRV/BamHI) and -278 (HincII/BamHI). The 141-bp 5'-UTR was cut out from all of them after adding two SacII sites by directed mutagenesis. The construction of these initial five human TPH2 promoter constructs is the work of Dr. Sylvie Lemonde. Two shorter constructs (-179 and -88) were produced by PCR amplification from the -278 construct (Figure 2.1). The primers for the -179 construct added a BglII site at the 5' end: 5'-CAC AGA TCT CCA AAA AGC TAC TCG ACC TAT G-3'. The primers for -88 construct added a XhoI site at the 5' end: 5'-ACT CTC GAG ATC GGC AAC CAG AAA TGA GT-3'. A common primer was used for the 3' end: 5'-GAT CTC CGT AAC GTG TAC ATC GAC TGA AAT CC-3'.

The rat Tph2 promoter region was cloned from genomic DNA obtained from L6 cells using the following primers: 5'-CTG TTA AGC CGC CAA GTA TCT GA-3' and 5'-TCC AAT TGT ACG AGT CCC AGA T-3'. The expected product was a 1227-bp segment spanning the region from -1004 to +223 from the start of transcription site. This product was cloned into a p-GEM-T-Easy vector (Promega) and used as a template to subclone a 1004-bp product containing the promoter

region from -1004 to +1 of the transcriptional start site. The product was subcloned by PCR amplification using primers that added BamHI sites to both ends: 5'-AAG GAT CCA ATT GTA CGA GTC CCA GAT T-3' and 5'-AAG GAT CCC CCT GGT GCT GAA GA-3'. The BamHI cohesive ends of the product were used to insert it into the BglII cloning site of the pGL3 Basic vector. This construction resulted in the -1004 construct and also the -1004flip construct, which has the insert in reverse orientation.

All plasmids were transformed into *E. coli* Dh5α, prepared by phenol / chlorophorm extraction and purified by ultracentrifugation in CsCl gradient. The A260 / A280 was over 1.8 in all cases. The sequence of all constructs was confirmed by automated sequencing in the Ontario Genomics Innovation Centre using the RV3 primer. For the experiments, all plasmids were diluted to 1 μg/μL in TE buffer (10 mM Tris / HCl and 1 mM EDTA, pH 8.0).

2.3 Cell culture

Human Embryonic Kidney 293 (HEK 293) and L6 rat skeletal muscle myoblasts were maintained at 37°C and a CO_2 level of 5%. Both cell lines were cultured in 10-cm Corning plates and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Wisent) supplemented with 8% fetal bovine serum (FBS, Wisent).

GH4C1, a cell line derived from a rat pituitary tumor, was grown at 37°C and at 5% CO2 level in Ham's F-10 medium (Wisent) with 8% FBS. These cells were cultured in 10-cm plates (Corning).

RN46A cells were maintained at 33°C and 5% CO_2 level. This cell line was grown in 10-cm Primaria plates (Falcon) and maintained in Neurobasal medium (Invitrogen) supplemented with 0.8 mM L-glutamine (Wisent) and 8% FBS.

Cultures were passaged 2-3 times a week using a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA in PBS.

Differentiating RN46A cells were grown in DMEM / F-12 medium (Wisent) supplemented with 1.5% FBS, 0.05 g/L Ovalbumin (SIGMA), 0.7 g/L Sodium Pyruvate (SIGMA), 100 IU Penicilin / 100 μg/mL Streptomycin mix (Wisent), 2 mM L-glutamine (Wisent) and 1% N-2 Supplement (Invitrogen). The differentiation process took place at 39°C and 5% CO_2 level for a period of 6 days.

2.4 Rat raphe primary cultures

2.4.1 Animals

Timed pregnant Sprague-Dawley rats were obtained from Charles River Inc. Rats were euthanized according to the experimental protocol approved by the University of Ottawa animal care facility (protocol # NSI-53). Rats were placed into a CO_2/O_2 chamber to lose consciousness, and then euthanized turning the O_2 level

to zero. On day E13, embryos were removed via c-section and placed in Hank's Balanced Salt Solution without Ca^{++} (HBSS, Wisent).

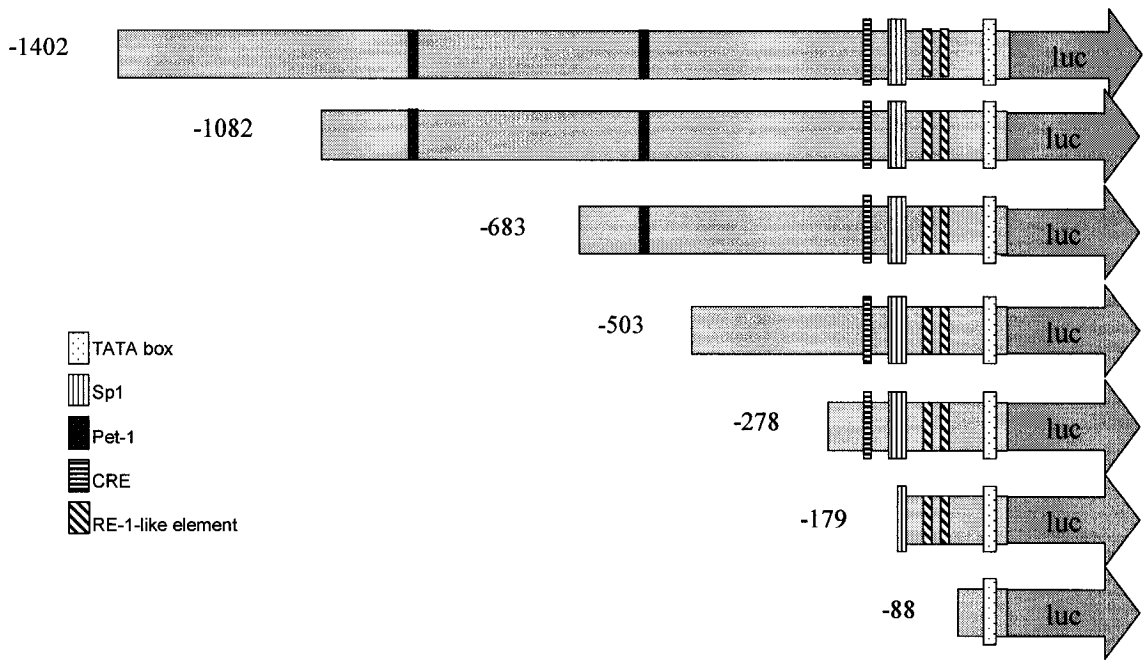

Figure 2.1 – Schematic representation of human TPH2 promoter deletion constructs

2.4.2 Dissection

Rostral raphe tissue was dissected from the midbrain according to the parameters suggested [Lautenschlager et al, 2000]. Dissection took place in HBSS without Ca^{++} (Wisent) under a Zeiss KL 1500 LCD dissecting microscope. Briefly, the heads were separated from the embryos' bodies and the meninges opened caudo-rostrally, to be gently removed from the midbrain/brainstem. The neural tube was opened ventrally and flattened. This neural tissue was reduced to a 0.5-mm-wide strip surrounding the midline of the rostral rhombencephalon [Konig et al, 1988]. In order to retain only serotonergic neurons in the B4 to B9 raphe nuclei, this strip was cropped rostrally and caudally.

2.4.3 Tissue culture

Raphe tissue was resuspended in 5 mL of HBSS without Ca^{++} and triturated by repeated pipetting. The homogenate was strained through an 80-μm pore mesh (BD Biosciences) to remove debris. At this point, an equal amount of HBSS with Ca^{++} was added. Cells were centrifuged 5 min at 500 g and the pellet was resuspended in 5 mL of Neurobasal media (Invitrogen) supplemented with 2% B-27 Supplement (Invitrogen), 5 mM L-glutamine (Wisent) and 100 IU Penicilin / 100 μg/mL Stretpomycin mix (Wisent).

Resuspended cells were plated on Primaria 6-well plates (Falcon) at a density of 3.5×10^6 cells/well. The plates were previously coated with 8 $\mu g/cm^2$ of

poly-D-lysine (Sigma). Primary cultures were incubated at 37°C at a 5% CO_2 level for 12 DIV. Every four days, 1 mL of medium from each well was replaced with fresh medium.

2.5 Reporter gene assays

2.5.1 Transient transfections

HEK 293, L6 and GH4C1 cells were cultured in 6-well plates (Corning) to attain a 50 to 60% confluence at the time of transfection. Differentiated RN46A cells, grown in Primaria 6-well plates (Falcon), were at least 90% confluent at transfection. Transfections were carried out using Lipofectamine Transfection reagent (Invitrogen) and Plus reagent (Invitrogen). The proportion of DNA to lipid and plus reagent was 1:1:1 in all transfections. The total amount of DNA per well was 1.5 μg, comprising 1 μg of luciferase reporter construct and 0.5 μg of pCMV-β-galactosidase plasmid. Briefly, the DNA was mixed with plus reagent in OptiMEM medium and pre-incubated for 15 min at room temperature, followed by addition of lipid and a second 15-min incubation. The transfection solution was added to the cells in 1 mL of serum-free, antibiotic-free OptiMEM medium (Invitrogen). The transfection took place for 3 h at 37°C, when it was stopped by adding 1 mL of maintenance medium. HEK 293, L6 and GH4C1 cells were collected at 48-54 h

after transfection. Differentiated RN46A cells were collected 24-30 h after transfection.

Primary culture neurons were transfected on DIV 10. Transfections were carried out using 1.5 μL of Lipofectamine 2000 (Invitrogen) and 1.5 μg total of DNA per well (1 μg of luciferase reporter construct and 0.5 μg of pCMV-β-galactosidase plasmid). The transfections took place for 3 h in antibiotic-free primary culture medium and the cells were collected after 48 h.

To calculate the efficiency of the transfection, primary cultures were transfected with GFP-containing plasmid. Conditions of transfection were maintained the same but 1 μg of GFP-containing plasmid was used instead of 1 μg of luciferase reporter construct. Efficiency was calculated as the proportion of transfected cells (GFP positive) to the total number of cells. Three fields in each of three wells were averaged. Cells were counted at 20X resolution using Axiovert S100 Zeiss microscope.

Ca^{++} mobilization was attained treating the transfected cells with the Ca^{++} ionophore ionomycin (Calbiochem). A 1 mg/mL stock solution was prepared by dissolving ionomycin in dimethyl sulfoxide (DMSO, Sigma). The treatment was applied to the cultures 24 h (RN46A cells) or 48 h (L6, GH4C1 or primary neurons) after the transfection. Final concentration of ionomycin was 0.7 μM. Controls were

treated with equal volume of DMSO. After 2 h of treatment, the medium was replaced for fresh medium and the cells collected 0, 2 or 4 h later.

2.5.2 Luciferase and β-galactosidase activity assays

Luciferase and β-galactosidase activities were assayed on cell lysates obtained from the transfected cells. To collect the cells, medium was sucked off and the transfected cells were rinsed with ice-cold PBS and scraped off the plates. The cells were transferred to a microtube and spun down for 5 min at 5000 rpm. The PBS supernatant was replaced with 200 μL of 1X Reporter Lysis Buffer (Promega). The cell pellet was resuspended by vigorous vortexing and lysates were immediately frozen at -80°C for at least 2 h. Cell lysates were thawed, cleared by vortexing and the debris was removed by spinning 10 min at maximum speed.

Luciferase activity was measured as the light intensity generated in the reaction luciferin + ATP → luciferyl adenylate + PP_i. For each reaction, 30 μL of lysate were mixed with 95 μL of luciferase reaction buffer which included the substrate luciferin (Molecular Probes). Reactions were set in 96 well Microlite 1+ microtiter plate (Thermo Labsystems). Luminescence was measured within two minutes of the start of the reaction using an LmaxII luminometer (Molecular Devices) at room temperature.

β-galactosidase activity was assayed by a colorimetric reaction that uses Chlorophenol Red β-D-galactopyranoside (CRGP, Calbiochem) as a substrate. β-galactosidase breaks down CRPG into chlorophenol red and β-D-galactopyranose,

thus changing the solution color from yellow to red. Each reaction included 30 μL of cell lysate and 200 μL of substrate-buffer mix. The buffer consisted of 0.25% v/v of β-ME and 1 mM MgSO4 in PBS. Substrate was diluted in buffer to a final concentration of 0.4 mg/mL. Reactions were set in 96-well transparent polystyrene microplates (Corning) and incubated at 37°C until the linear range of the reaction was reached. Absorbance was read at 595 nm in a SpectraMax M2 Multi-Detection Microplate Reader (Molecular Devices) at room temperature.

Luciferase values were normalized to β-galactosidase values of the same transfection reaction. Replicates were averaged and referenced to the average ratio obtained for pGL3 B vector.

2.6 5' Rapid amplification of cDNA end (5'-RACE)

5' Rapid amplification of cDNA end was performed to uncover the presence of different transcriptional start sites in the human TPH2 gene. 5'-RACE makes use of a cDNA library where every template molecule has an adaptor oligonucleotide attached to its 5' end. A PCR reaction using a primer specific to the target gene and a second primer that binds to the adaptor allows the amplification of all possible 5' variants of the gene.

The procedure was done using the Marathon-Ready cDNA kit (BD Biosciences) following manufacturer's protocol. In this case, a human whole-brain cDNA library was screened to identify possible 5' variants in the TPH2 gene. The

commercially available library is prepared by reverse transcription of poly A+ RNA obtained from normal whole brains pooled from eight Caucasian males. The 5'-adaptor primer (AP-1) sequence is 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3', while the TPH2-specific primer was 5'-TTT GGA GAG CTC CCG GAA TAC AAC ACC C-3'. The 5'-RACE PCR program was: 94°C for 30 s, four cycles of 94°C for 5 s and 72°C for 4 min, four cycles of 94°C for 5 s and 70°C for 4 min and finally 24 cycles of 94°C for 5 s and 68°C for 4 min.

A second PCR was performed using as template the product of the first reaction. The product was diluted 50 times in nuclease-free water. This nested PCR involved another internal gene-specific primer and a second 5'-adaptor primer (AP-2). AP-2 sequence is: 5'-ACT CAC TAT AGG GCT CGA GCG GC-3', and the TPH2-specific nested primer was 5'-AAC CAG GGC ACA TCC TCT AGC TCT TCT TCC-3'. As a positive control, a reaction was set in which AP-2 was replaced by a sense TPH2-specific primer of sequence 5'-CTG GCA AAA ATG ACG ACA AAG GCA ACA AG-3'. The expected product size amplified by the two nested TPH2 primers was 340 bp. The nested PCR program was: 94°C for 30 s followed by 24 cycles of 94°C for 5 s and 72.5°C for 3 min.

The products of both PCR reactions were run in agarose gels and tested for the presence of TPH2 bands by Southern blot. Positive bands resulting form the nested PCR were gel-purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's protocol. Purified bands were cloned into p-GEM-T-Easy vector (Promega) and sequenced by automated fluorescence method.

2.7 Southern Blot

Amplification products from the 5'-RACE and the nested PCR were screened by Southern blot using two human TPH2-specific probes. One probe targeted the 5'-UTR and the other one targeted the coding sequence. The 5'-UTR probe was obtained by cutting a 151-bp-long MspI/MspI fragment from the -683 construct. The CDS probe was 339 bp long and was obtained by PCR amplification from a human cDNA template. The probe spans from base 263 to base 602 of the cDNA, including regions of exons 2, 3 and 4. Both probes were tested against negative and positive control before being used to test the 5'-RACE products.

The probes were labelled with ^{32}P-dCTP (Amersham Biosciences) using the Rediprime II Random PrimeLabelling System (Amersham Biosciences) according to manufacturer's protocol. The radioactive probes were purified by passing through a sephadex G-100 column. The final probe concentration was 0.45 ng/μL and the specific activity ranged between $1x10^8$ and $3x10^8$ cpm/μg.

The PCR products were run in a 1.5% agarose gel. The gel was washed with denaturing solution (1.5 M NaCl and 0.5 M NaOH) and then neutralized (1.5 M NaCl and 1 M Tris). The denaturalized DNA was transferred overnight to a Hybond-N+ membrane (Amersham Biosciences). The membrane was blocked for 30 min with salmon sperm DNA in the Ultra-hyb hybridization buffer (Ambion). The buffer was replaced for fresh one and the probe was added. Hybridization took place overnight at 42°C. After washing, the membrane was exposed to BioMax MR Film (Kodak) for 4 h.

2.8 Quantitative real-time RT-PCR

2.8.1 RNA extraction and DNAse treatment

Total RNA was obtained from cells contained in one well of a 6-well plate. Wells were washed twice with cold PBS to get rid of dead cells. Remaining cells were lysed with 200 μL of TRIzol (Invitrogen) for 5 min. The resulting lysate was extracted at room temperature using 200 μL of chlorophorm for 2 min. Following 15-min spin at 4°C, the upper, aqueous phase was carefully transferred to a fresh new tube for isopropanol precipitation overnight at -20°C. The pellet was washed with ethanol 70% and resuspended in 25 μL of DEPC-treated water. RNA yields, as quantified by spectrophotometry at 260 nm, ranged from 0.3 μg/μL to 1.0 μg/μL.

To avoid genomic DNA contamination at the PCR step, samples were treated with DNAse using Turbo DNA-free kit (Ambion) according to manufacturer's protocol. Briefly, 1 μL of Turbo DNAse was applied to 5 μg of RNA and diluted to a final volume of 50 μL. The mixture was incubated for 30 min at 37°C. After inactivation and pelleting of the enzyme, RNA was quantified by spectrophotometry at 260 nm. Concentrations ranged from 0.07 to 0.10 μg/μL and 260 / 280 ratios were always over 1.8.

2.8.2 Reverse transcription

Reverse transcription was carried out on 0.5 μg of DNAse-treated RNA in 20-μL reactions. Reaction mix was assembled in two steps. First, 2 μL of random decamers (Ambion) as primers, 4 μL of 10 mM dNTP mix, 0.5 μg of RNA and DEPC-treated water up to 16 μL were heated for 3 min at 70°C. After cooling down on ice, 4 μL containing 10X first-strand buffer (Ambion), 0.25 μL of M-MLV reverse transcriptase (Ambion) and 0.25 μL of RNAse inhibitor (Promega) were added. At this time, parallel reactions were set lacking M-MLV enzyme to serve as no-reverse transcription controls. Reverse transcription took place for 60 min at 42°C. The M-MLV enzyme was inactivated by 10-min treatment at 95°C. Newly synthesised cDNA was stored at -20°C.

2.8.3 Quantitative real-time PCR

2.8.3.1 TaqMan chemistry

TaqMan chemistry allows one to follow a PCR in real time by measuring the fluorescence emitted by the products after each reaction cycle. A gene-specific oligonucleotide (the TaqMan probe) anneals to the sequence between the gene-specific primers. The probe is tagged with a fluorescent dye at the 5' end, but also with a quencher at the 3' end that absorbs the energy emitted by the former.

Fluorescence will only be detected when the DNA polymerase cleaves the probe, and releases the fluorescent tag far from the quencher.

Probes and primers for the target gene were purchased as a Gene Expression kit (Applied Biosystems). Product number is Rn00598017_m1 for Tph2 gene. GAPDH expression was measured using Pre-developed Taqman Assay Reagent for rat GAPDH. The Tph2 probe is FAM-labelled, while the GAPDH probe is VIC-labelled. GAPDH was used as internal control and its probes were included in every reaction. PCR was performed in 10-μL reactions. Each reaction carried 5 μL of Universal PCR Master Mix (Applied Biosystems), 0.5 μL of FAM-labelled probe, 0.5 μL of VIC-labelled probe, 3 μL of nuclease-free water and 1 μL of sample.

Standard curves were prepared by serial dilution of a plasmid carrying the full length CDS of each gene. All samples were run in duplicates in a Rotor Gene 3000 cycler (Corbett Research). Two no template controls for each gene were included in each run. In addition, one point of the standard curves was run in duplicate along with the samples. The cycling program used was: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

2.8.3.2 SYBR Green I chemistry

SYBR Green I dye fluoresces when bound to double-stranded DNA, and therefore the intensity of signal follows the amount of product in real time.

Specific primers were designed to produce rat Tph2 and GAPDH amplicons. Tph2 primers were 5'-TAA ATA CTG GGC CAG GAG AGG-3' for the sense orientation and 5'-AGT GTC TTT GCC GCT TCT CTT-3' for the antisense. GAPDH primers were 5'-AAG TTC AAC GGC ACA GTC AAG-3' for the sense direction and 5'-ACA TAC TCA GCA CCA GCA TCA C-3' for the reverse orientation. The expected Tph2 product spanned an intron/exon boundary and was 130 bp long, while the GAPDH amplicon was 121 bp. PCR was performed in 25-μL reactions. Each reaction carried 12.5 μL of 2X Buffer (BD Biosciences) and 0.25 μL of 50X PolyQ Taq polymerase (BD Biosciences), 0.25 μM of sense primer, 0.25 μM of antisense primer, 1 μL of SYBR Green solution, 6.75 μL of nuclease-free water and 2 μL of sample.

Tph2 standard curve was prepared by serial dilution of a p-GEM-T-Easy vector carrying the 130-bp amplicon. GAPDH standard curve was generated using the same dilutions used for the Taqman curve. All samples were run in duplicates in a Rotor Gene 3000 cycler (Corbett Research). Two no template controls for each gene were included in each run. In addition, one point of the standard curves was run in duplicate along with the samples. The cycling program used was: 95°C for 3 min followed by 45 cycles of 92°C for 20 s, 58ºC for 20 s, 72ºC for 20 and 82ºC for 10 s. After the last cycle, the program held 72ºC for 15 min. To test the specificity of the PCR products, a melting curve was generated after each run (1ºC every 5 s from 72 to 99ºC).

In order to confirm their identity, the real time PCR products were cloned into p-GEM-T-Easy vector (Promega) and sent for automated sequencing from the T7 promoter primer to the Ontario Genomics Innovation Centre.

2.8.4 Treatments

For Ca^{++} mobilization, cells were treated with KCl (EMB) for either 1 or 3 h before RNA collection. KCl was dissolved in a small volume of culture medium that was added to the cells to attain a final concentration of 10, 20 or 40 mM above the normal medium K^{+} concentration (5.6 mM). Alternatively, cells were treated with ionomycin (Calbiochem) for 3 h. The ionomycin, prepared in DMSO, was dissolved in a small volume of culture medium and added to the cells to reach a final concentration of 1.4 μM. Controls were treated with equal volume of DMSO.

To test the mechanism of action of KCl on the RN46A cells, transcriptional-inhibitor Actinomycin D (Sigma) was used. A stock solution of Actinomycin D was prepared in DMSO at a concentration of 1 mg/mL. For the experiments, a small volume was added to the cultures 15 min before the addition of KCl (i.e. 75 minutes before RNA collection) to reach a final concentration of 2.5 μg/mL. Controls were treated with equal volume of DMSO.

Other experiments to devise KCl action included treatment with the L-type Ca^{++} channel blocker Nifedipine (Sigma), the extracellular Ca^{++} chelator ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma) and the intracellular Ca^{++} chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,-N',N'-tetraacetic

acid tetraacetoxy-methyl ester (BAPTA-AM, Sigma). Stock solutions of Nifedipine and BAPTA-AM were prepared in DMSO at concentrations of 10 and 20 mM, respectively. Both reagents were dissolved in a small volume of culture medium and added to the cells 30 min before the addition of KCl (i.e. 90 minutes before RNA extraction). Controls were treated with equal volume of DMSO. Nifedipine was prepared to a final concentration of either 1 or 10 μM. BAPTA-AM was prepared to a final concentration of either 2 or 10 μM. EGTA was dissolved directly in a small volume of culture medium and added to the cells 5 min before addition of KCl (i.e. 65 min before RNA extraction) to reach a final concentration of 2 mM.

CHAPTER III – RESULTS

3.1 Cloning and sequence analysis of the human TPH2 promoter region

Work previous to my arrival to the Albert's Lab resulted in the isolation and cloning of a 5.1 kb fragment that spanned the 5' flanking region of the human TPH2 gene, including the reported 5'-UTR. Sequencing of this DNA fragment showed that it corresponds to the published sequence.

The putative human TPH2 promoter region was scanned with different bioinformatic tools to identify potential regulatory elements. The rat Tph2 5' upstream region was analyzed as well to identify conserved motifs.

Firstly, a 6 kb fragment of genomic DNA spanning from positions -5073 to +879 of the reported transcriptional start site (TSS) was run through Proscan Version 1.7, a software that predicts eukaryotic promoters and putative transcription factor binding sites (TFBS) based on homology to previously reported promoters [Prestridge, 1995]. The scan resulted in three putative promoter regions, one of them fully coincident with the expected one. The Proscan software predicted the TSS and found a consensus TATA box. It also found several other elements similar to known TFBS (Table 3.1).

Secondly, the region between -5062 and +141 was put through the Transcription Element Search Software (TESS) [Schug and Overton, 1997]. Numerous TFBS were found across the sequence, but only those located in the

first 500 bp proximal to the TSS that achieved a L_a (log-likelihood) score higher than 14 are listed in table 3.2. The parameter L_a is an index of the homology between the scanned sequence and the model sequences found in the TRANSFAC database. This analysis found a TATA box in the appropriate position and several sites that might bind Ca^{++}-dependent transcription factors, like cAMP-responsive element binding protein (CREB), c-Jun, CCAAT/enhancer binding protein, isoforms α and β (C/EBP) and the myocyte enhancer factor-2 (MEF-2). Interestingly, many of these coincided with the Proscan results, such as the TATA box at position -25, several overlapping Sp1 elements located around position -172 and a CRE site at position -235. These elements are marked in the diagrams depicting the deletion constructs (Figure 2.1).

Like coding sequences, genomic DNA with a regulatory function is subject of evolutionary constraints [Dermitzakis and Clark, 2002]. For this reason, promoter sequences are expected to be fairly conserved among related species. Alignment of rat and human 5' flanking regions of the TPH2 gene using Blast 2 Sequences software [Tatusova and Madden, 1999] demonstrated homology greater than 83% in the first 200 bp upstream of the reported TSS. Similar results were obtained when the sequences were aligned using MegAlign software (Figure 3.1). These results indicate that DNA elements functionally relevant are located within 200 bp of the TSS. However, they do not give any definitive information about the existence of other TFBS further upstream.

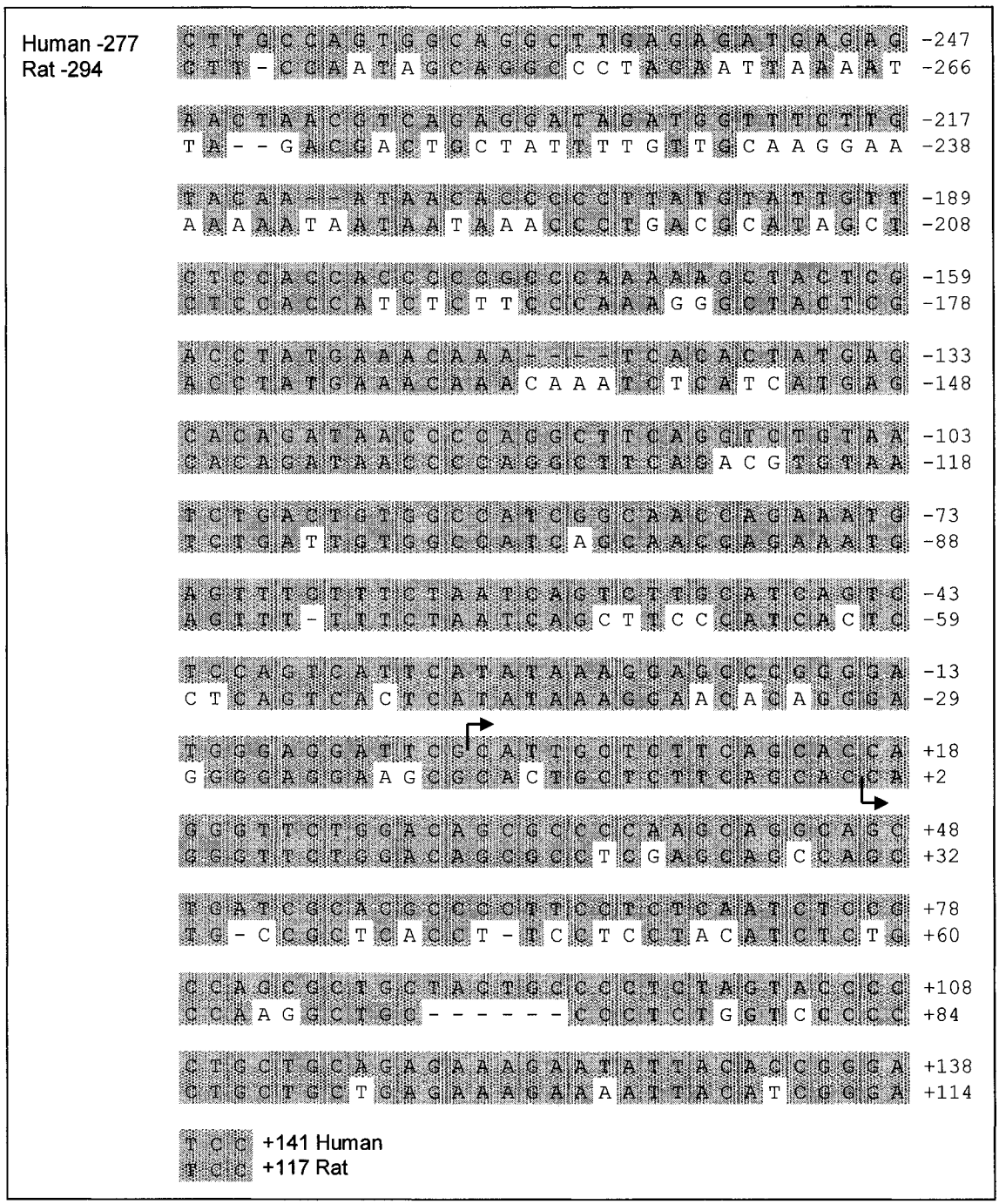

Figure 3.1 – ClustalW alignment of human and rat genomic DNA sequences from -277 and -294, respectively, to the end of the 5'-UTR, performed with MegAlign software (Lasergene) in the slow/accurate mode. Grey shadow indicates homology. Arrows indicate reported transcription start sites.

3.2 Experimental confirmation of human TPH2 transcriptional start site

To confirm that the cloned sequence corresponds to the 5' flanking region of the TPH2 gene, a 5'-Rapid Amplification of cDNA Ends (5'-RACE) was carried out. In this case, a human whole-brain library from a Marathon-Ready kit (BD Biosciences) was used as template. A faint PCR product was seen around 900 bp (Figure 3.2a), in agreement with the expected size that would be obtained from the predicted TSS (890 bp). Furthermore, this was the only band that hybridized to a Southern blot probe made out of portions of exons 2, 3 and 4 and another from the 5'-UTR. The absence of larger bands is an indication that there are no alternative TSS further upstream that added an exon, resulting in a larger product. No product was seen in the no-template control and the expected 1.2 kb band was seen when a human GAPDH primer was used (Figure 3.2a). A PCR performed in less stringent primer-annealing conditions showed the same single band, while in more stringent conditions there was no product (not shown). A dilution of the first PCR product was used as template of a second, nested PCR. At least six bands ranging from less than 200 to 650 bp were seen in the agarose gel, and all of them hybridized to a probe from exons 2 to 4, while only the larger ones reacted to a 5'-UTR probe (Figure 3.2b). None of them was larger than the maximum expected size for the reported of 640 bp, suggesting no alternative upstream TSS went undetected in the first PCR. The bands were individually purified, resulting in eleven clones that could be grouped in only four distinct sequences (Figure 3.2c). Three clones showed the complete expected sequence from +1 (total product size of 633 bp), while four showed sequences from position +144 (490 bp), three other

started at +414 (219 bp) and only one presented a short sequence from +457 (176 bp). Most likely, shorter products originated not as consequence of alternative TSS but rather because of mRNA degradation (RNA was extracted from human brain samples) or incomplete reverse transcription, two main caveats of 5'-RACE experiments [Matz et al, 2003].

3.3 Endogenous expression of Tph2

The model systems used were tested by RT-PCR to verify the endogenous expression of Tph2 mRNA. Primers were designed to span a region from exon 1 to exon 2. As expected, primary cultures showed strong expression of Tph2 mRNA, while differentiated RN46A cells expressed noticeable less. L6 myoblasts were negative and thus used as negative control in further experiments. Pituitary GH4C1 cells, a non neuronal cell line, turned out to express Tph2 mRNA. Plasmids carrying the full length CDS of Tph2 or GAPDH served as positive controls and reactions without template were used as negative controls (Figure 3.3).

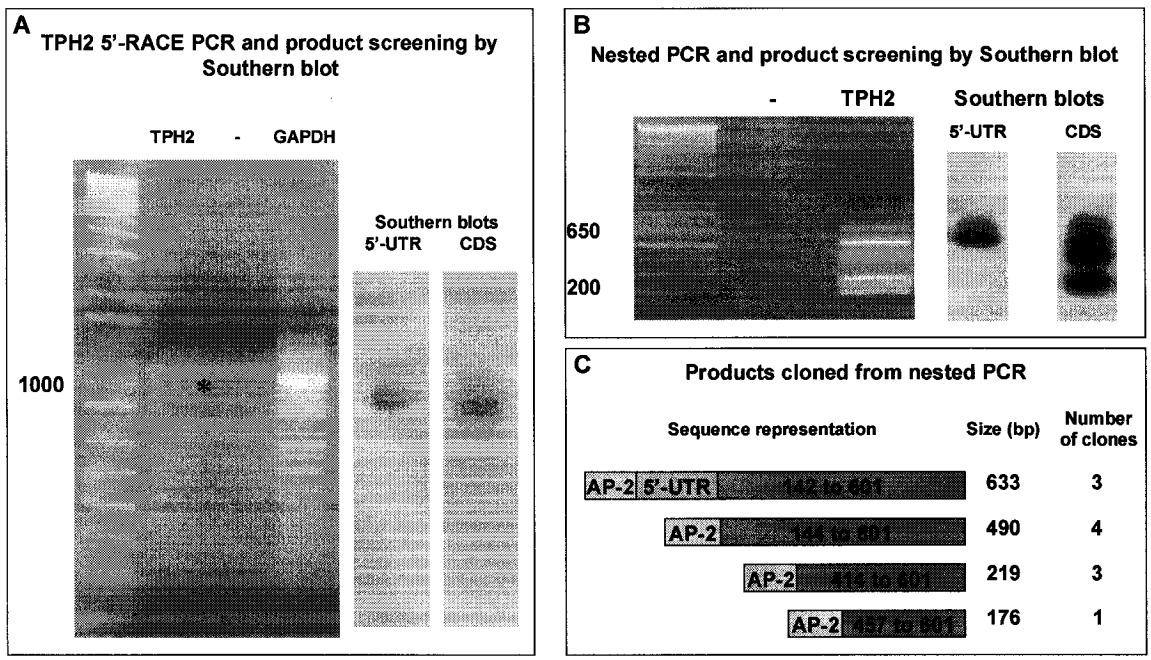

Figure 3.2 - Identification of transcriptional start site. **A)** TPH2 5'-RACE PCR and product screening by Southern blot. A whole-brain human cDNA library was screened with a specific TPH2 primer (lane 1), showing a band around 900 bp (star). As negative control, template was not included in the PCR mix (lane 2). As positive control, the library was screened with a specific GAPDH primer (lane 3). The TPH2 band was recognized by Southern blots using probes spanning the TPH2 5'-UTR or parts of the CDS. **B)** Nested PCR and product screening by Southern blot. Diluted 5'-RACE PCR product was amplified with a nested TPH2 primer (lane 2). As negative control, template was not included in the PCR mix (lane 1). The bands were recognized by Southern blots using probes spanning the TPH2 5'-UTR or parts of the CDS. **C)** Schematic representation of the four species cloned from the isolated bands, numbers in the diagram refer to nucleotide position in the cDNA sequence. *AP-2* stands for adaptor primer-2.

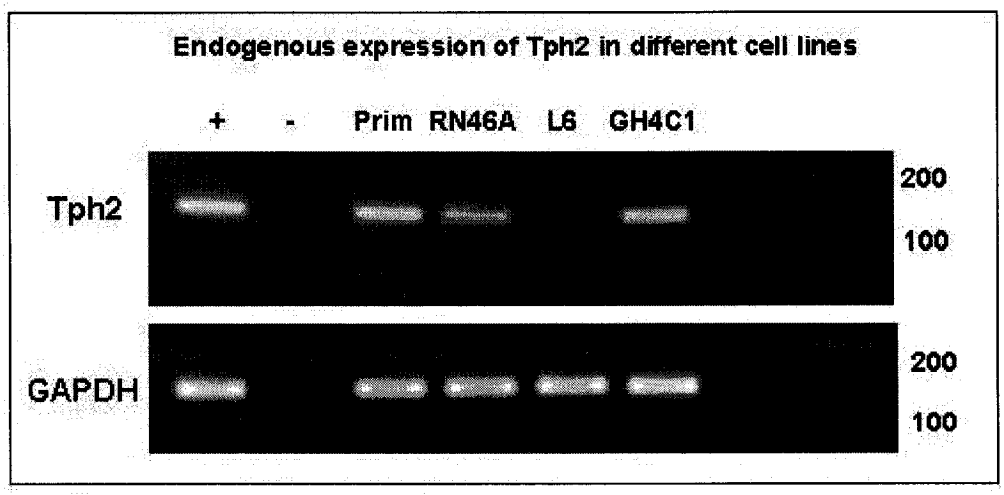

Figure 3.3 - Endogenous expression of Tph2 mRNA in different cell lines, detected by RT-PCR. *Prim* indicates rat raphe primary neurons. Positive controls are plasmids carrying the corresponding gene, negative controls lack template in the PCR mix.

3.4 Transcriptional activity of the TPH2 promoter

3.4.1 Transcriptional activity of the human TPH2 promoter

Seven deletion constructs of different length carrying the putative human TPH2 promoter were subcloned into luciferase reporter vectors (Dr. Sylvie Lemonde prepared five of them) (Figure 3.4).

The plasmids bearing the human promoter region were transfected into a neuronal, serotonergic cell line (differentiated RN46A, figure 3.4a) and two other Tph2-negative cell lines, one human (HEK 293, figure 3.4b) and one of rat origin (L6, figure 3.4c). Relative luciferase activity of the promoter constructs was significantly higher than the control (empty vector) in all cell lines, indicating that the sequence cloned has transcriptional activity. In particular, the shortest construct (-88) promoted transcription of the reporter gene 2.5 times more than the empty vector in RN46A (t-test $p=0.019$) and L6 cells (t-test $p<0.001$), and 3.0 times more in HEK 293 cells (t-test $p<0.001$). The activity of the shortest construct was also significantly higher than that of the -179 construct ($p<0.05$ for all cell lines), suggesting the location of a repressor binding element in the region. This result is in agreement with the reported finding of a bipartite RE-1-like sequence around 130 bp upstream of the origin of transcription [Mueller and Patel, 2005]. This may imply that the REST transcription factor (or a related protein) might be involved in repression of TPH2, as it does in many other neuronally-restricted genes [Jones and Meech, 1999].

In non-serotonergic HEK 293 cells, the transcriptional activity remained low for larger constructs. On the other hand, activity in differentiated RN46A and L6 cells increased significantly for larger constructs, implying the presence of an activating sequence downstream of position -503. In RN46A cells, the activity stabilized at a level around 2.3 times the control for all constructs longer than -278, suggesting that enhancing elements are present in the proximal promoter region. Similarly, activity of longer constructs in L6 cells was also higher than the -179 construct. In this cell line, activity of longer constructs was on average around 3 times the empty vector.

The TPH2 promoter is also active in raphe primary neurons (Figure 3.5). In these cells, the transcriptional activity of the -278 construct was 1.7 times higher than that of the empty vector (t-test $p=0.005$). Interestingly, this value is in agreement with that obtained in differentiated RN46A cells, the other model of serotonergic neurons.

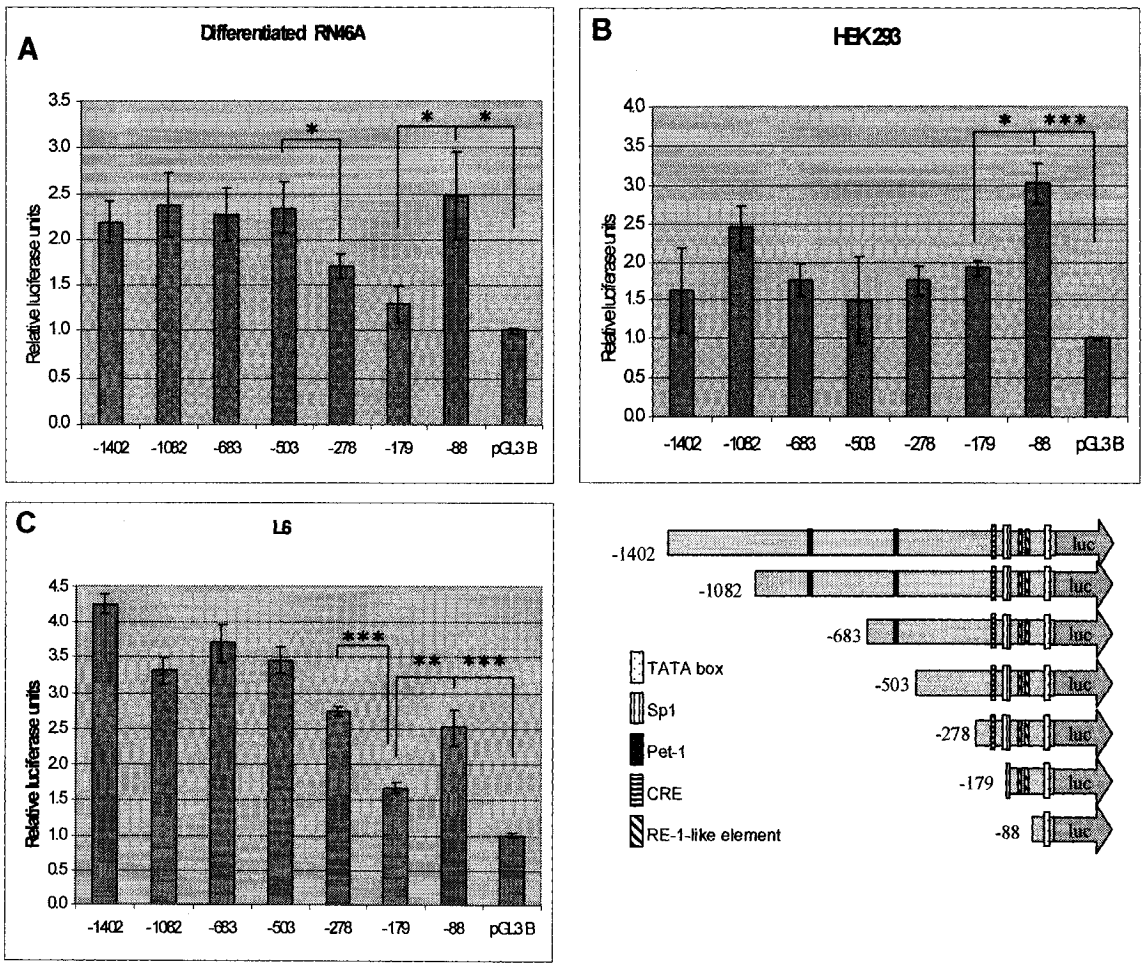

Figure 3.4 - Transcriptional activity of the human TPH2 promoter in different cell lines, as measured by luciferase reporter assays of deletion constructs. Relative luciferase activity is luciferase activity normalized to β-galactosidase activity in the same culture, referenced to activity of empty vector (pGL3 B). Data shown as mean +/- SEM. **A)** Differentiated RN46A cells, constructs -1402 (n=6), -1082 (n=7), -683 (n=7), -503 (n=7), -278 (n=19), -179 (n=3) and -88 (n=3). Relative luciferase activity of pGL3 B was 0.120 +/- 0.013. **B)** HEK 293 cells, constructs -1402 (n=2), -1082 (n=6), -683 (n=2), -503 (n=3), -278 (n=9), -179 (n=3) and -88 (n=5). Relative luciferase activity of pGL3 B was 2.320 +/- 0.070. **C)** L6 cells, constructs -1402 (n=5), -1082 (n=9), -683 (n=6), -503 (n=6), -278 (n=3), -179 (n=6) and -88 (n=5). Relative luciferase activity of pGL3 B was 1.944 +/- 0.101.

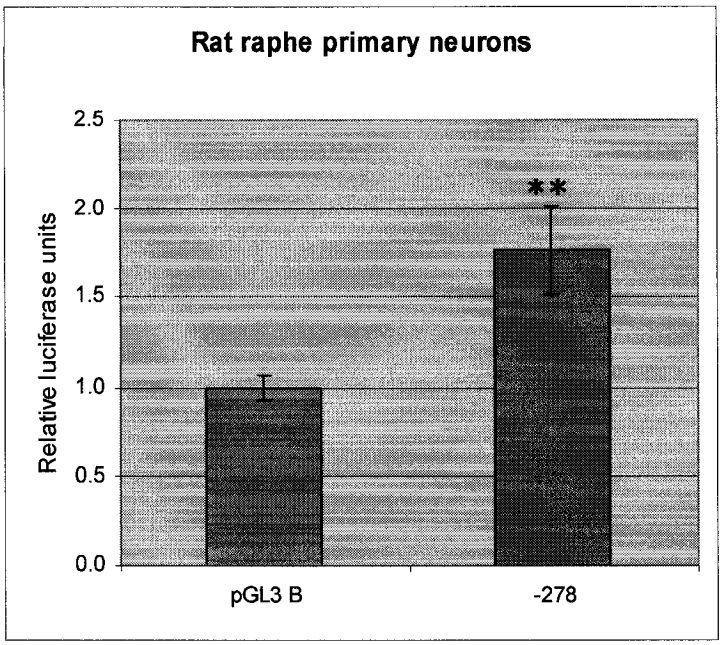

Figure 3.5 - Transcriptional activity of the human TPH2 promoter in rat raphe primary neurons, as measured by luciferase reporter assays of the -278 deletion construct (n=10, t-test p=0.005). Relative luciferase activity is luciferase activity normalized to β-galactosidase activity in the same culture, referenced to activity of empty vector (pGL3 B). Data shown as mean +/- SEM. Relative luciferase activity of pGL3 B was 0.642 +/- 0.083.

3.4.2 Transcriptional activity of the rat Tph2 promoter

In spite of the high homology observed between rat and human promoters (83% in the first 200 bp, but lost further upstream), it is possible that small sequence differences may have prevented strong activity of the human constructs in the Tph2-positive rat cell line. To test this hypothesis, a 1004-bp-long fragment from the rat promoter region was cloned into pGL3 B and transfected into various cell lines (Figure 3.6). The activity of this construct was 2.8 times that of the basal in differentiated RN46A cells (t-test $p=0.002$) and 2.9 times in raphe primary neurons (t-test $p=0.002$), a level not substantially higher than that obtained with human constructs. As seen with the human promoter, the luciferase activity obtained from the rat promoter was similar in both models of serotonergic cells. In L6 cells, the rat promoter activity was 4.5 times that of the empty control (t-test $p<0.001$), again not showing a substantial increase compared to the human counterparts. In GH4C1 cells, another Tph2-positive cell line, activity of the rat promoter was 8.0 times the basal (t-test $p=0.019$).

In addition, in all cell lines, the transcriptional activity of a reporter construct containing the rat promoter in an antisense orientation was significantly lower than the empty vector, further demonstrating the presence of a promoter in the 5' flanking region of the Tph2 gene (Figure 3.6).

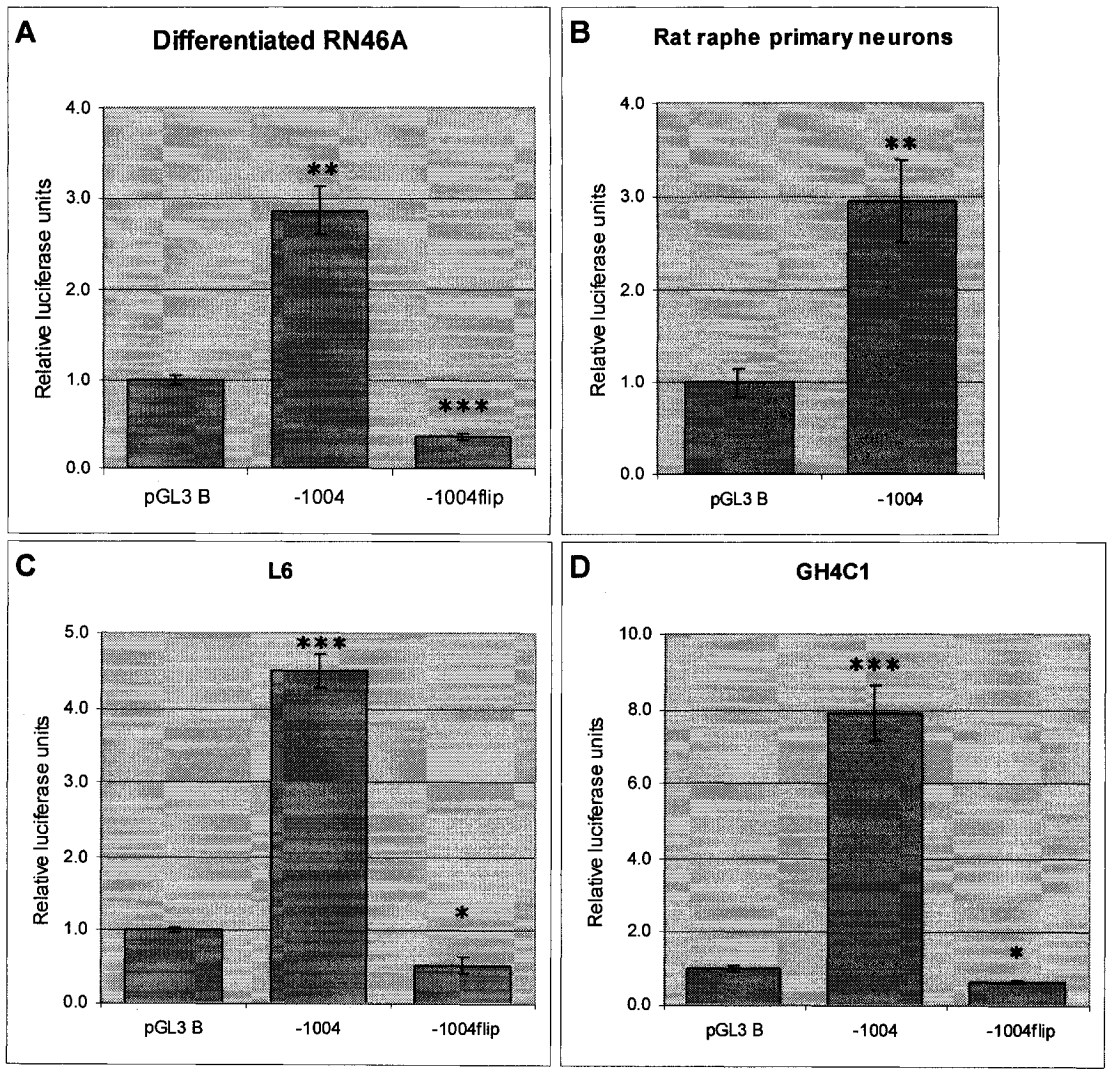

Figure 3.6 - Transcriptional activity of the rat Tph2 promoter in different cell lines, as measured by luciferase reporter assays. Relative luciferase activity is luciferase activity normalized to β-galactosidase activity in the same culture, referenced to activity of empty vector (pGL3 B). Data shown as mean +/- SEM. -1004flip indicates an antisense -1004 construct. Stars (*) indicate statistical comparison against pGL3 B. **A)** Differentiated RN46A cells, constructs -1004 (n=7, t-test p=0.002) and -1004flip (n=4, t-test p=0.001). Relative luciferase activity of pGL3 B was 0.272 +/- 0.056. **B)** Rat raphe primary neurons, construct -1004 (n=8, t-test p=0.002). Relative luciferase activity of pGL3 B was 0.848 +/- 0.109. **C)** L6 cells, constructs -1004 (n=8, t-test p<0.001) and -1004flip (n=3, t-test p=0.014). Relative luciferase activity of pGL3 B was 1.579 +/- 0.023. **D)** GH4C1 cells, constructs –1004 (n=6, t-test p<0.001) and -1004flip (n=3, t-test p=0.019). Relative luciferase activity of pGL3 B was 1.872 +/- 0.093.

3.5 Ca^{++}-induced activation of TPH2 promoter activity

Responsiveness to ionomycin, a Ca^{++}-mobilizing agent, was tested in differentiated RN46A cells transfected with the TPH2 promoter constructs. Paired transfected cultures (i.e. transfected at the same time with the same transfection mix) were treated for 2 h with either ionomycin 0.7 μM or vehicle, washed and finally collected 4 h later. Ionomycin treatment caused in average a 1.6-fold increase in TPH2 promoter-driven transcription (Figure 3.7). For instance, transcriptional activity of the -88 construct in ionomycin-treated cells was 46.4% higher than in vehicle-treated cells. On the other hand, relative luciferase activity of the empty vector changed 8.1%. The 46.4% increase observed with the promoter is significantly higher than the 8.1% observed for the control (t-test p=0.003). Longer constructs showed up to 69% increase in activity, but in no case it was significantly higher than the increase seen for the -88 construct. In other words, all constructs tested were capable of expressing Ca^{++} responsiveness and therefore it can be assumed that the responsible DNA element lies in the proximal 88 base pairs upstream of the transcriptional start site.

To further characterize this response, the -278 construct (that gave the most consistent response) was tested to see if it could drive Ca^{++}-induced transcription in different cell lines. Interestingly, the construct only responded in serotonergic, neuronal RN46A cells. Ionomycin-induced activity of this construct was 52.7% higher than the activity of vehicle-treated controls (Figure 3.8a). Again, this increase was significantly higher than the 8.1% change seen for the empty vector

(t-test $p<0.001$). On the other hand, ionomycin treatment in non neuronal cells like L6 myoblasts (Tph2-negative) or pituitary GH4C1 cells (Tph2-positive) was not capable of activating the -278 promoter construct, suggesting only neuronal serotonergic cells may have the required proteins for the induction.

Similar results were obtained when the rat Tph2 promoter was tested. The -1004 construct bearing the rat sequence was transfected in differentiated RN46A cells, where transcriptional activity increased 28.3% after Ca^{++} mobilization (Figure 3.8b). This increase was significantly higher than the 8.3% change seen for the empty vector (t-test $p=0.008$). In L6 or GH4C1 cells, the rat promoter could not be activated by the ionomycin treatment. Since the rat and human promoter are more the 80% homologous in the first 90 bp upstream of the TSS, it is likely that the element driving these similar responses may be the same.

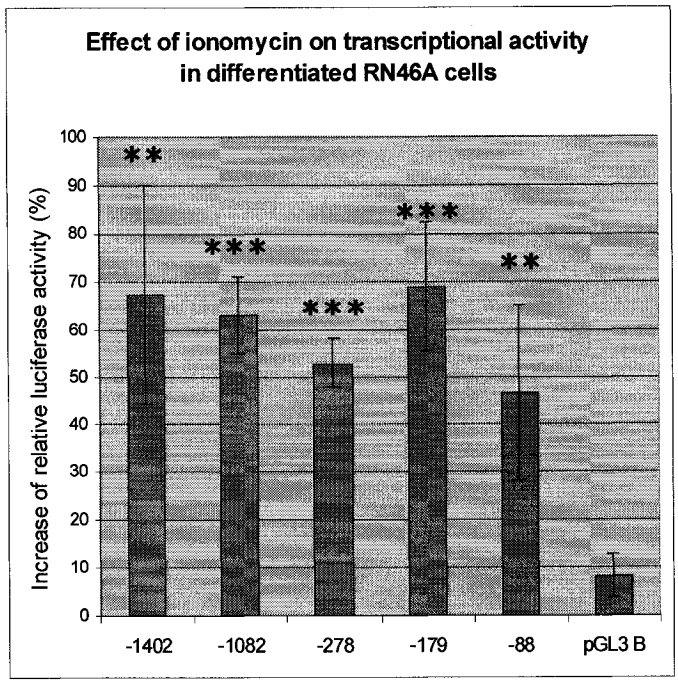

Figure 3.7 - Effect of ionomycin on transcriptional activity of the human TPH2 promoter in differentiated RN46A cells, as measured by luciferase reporter assays. Relative luciferase activity is luciferase activity normalized to β-galactosidase activity in the same culture. Transfected cultures were treated with ionomycin 0.7 μM or vehicle for 2 h, then washed and collected 4 h later. Samples were paired at transfection, then treated and the treated / untreated ratio was calculated for each pair. The increase in relative luciferase units was measured as % above untreated. Data shown as mean +/- SEM. Stars (*) indicate statistical comparison against change seen in pGL3 B. Effect of ionomycin for constructs -1402 (n=6, t-test $p=0.001$), -1082 (n=6, t-test $p<0.001$), -278 (n=10, t-test $p<0.001$), -179 (n=5, t-test $p<0.001$) and -88 (n=3, t-test $p=0.003$).

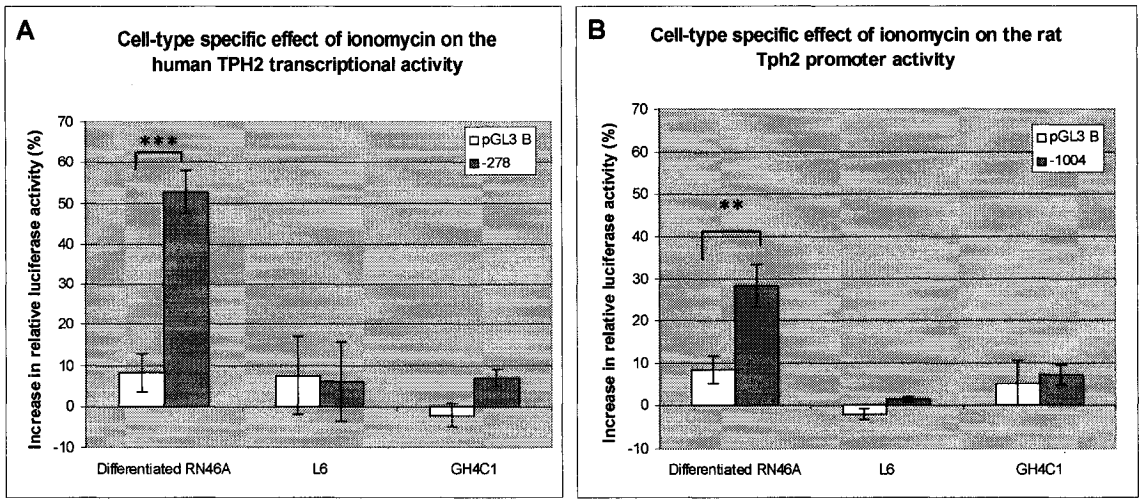

Figure 3.8 - Cell-type specific effect of ionomycin on transcriptional activity of the Tph2 promoter. Relative luciferase activity is luciferase activity normalized to β-galactosidase activity in the same culture. Cultures were transfected with a promoter construct, treated with ionomycin 0.7 μM or vehicle for 2 h, then washed and collected 2 or 4 h later. The increase in relative luciferase units was measured as % above untreated. Data shown as mean +/- SEM. **A)** Effect of ionomycin on transcriptional activity of the human -278 TPH2 promoter construct, in differentiated RN46A cells (n=10, t-test $p<0.001$), L6 (n=6, t-test NS) and GH4C1 (n=3, t-test NS). **B)** Effect of ionomycin on transcriptional activity of the rat -1004 Tph2 promoter construct, in differentiated RN46A cells (n=10, t-test $p=0.008$), L6 (n=6, t-test NS) and GH4C1 (n=3, t-test NS).

3.6 Ca^{++}-induced increase in endogenous Tph2 transcription

3.6.1 Effect of KCl or ionomycin on endogenous Tph2 mRNA levels

To test whether Ca^{++} mobilization affected also endogenous Tph2 expression, differentiated RN46A cells were treated with KCl or ionomycin and levels of Tph2 mRNA were measured by real-time quantitative RT-PCR. Changes in extracellular concentration of K^+ alter the cell membrane potential, opening voltage-sensitive Ca^{++} channels that allow influx of Ca^{++} into the cells. Ionomycin is an ionophore that allows permeation of Ca^{++} ions through the membrane and also release from intracellular Ca^{++} stores.

Addition of KCl to the cell culture increased mRNA levels in a time- and concentration-dependent manner (Figure 3.9a and b). Treatment of differentiated RN46A cells in culture for 1 h with 10 mM KCl did not increase normalized mRNA levels over the control, but treatment using 20 or 40 mM KCl elevated mRNA levels 3.2 (t-test $p=0.002$) and 13.2 times (t-test $p=0.001$), respectively. In a separate experiment, the K^+-induced increase in mRNA concentration was shown to be time-dependent. While treating the cells with 40 mM KCl for 1 h elevated Tph2 mRNA 10.4 times over the control (t-test $p<0.001$), a 3-h treatment only increased Tph2 mRNA 4.0-fold (t-test $p<0.001$).

Consistent with these results, treatment of differentiated RN46A cells with ionomycin also induced Tph2 mRNA (Figure 3.9c). When treated for 3 h with ionomycin 1.4 μM, Tph2 mRNA levels increased by 3-fold (t-test $p=0.027$). On the

other hand, treatment with 0.14 μM ionomycin did not stimulate Tph2 expression levels.

Taken together, these results suggest that Ca^{++} mobilization increases Tph2 expression in differentiated RN46A cells.

3.6.2 Effect of actinomycin D on KCl-induced increase in Tph2 mRNA levels

Actinomycin D interferes with the elongation of nascent mRNA during transcription. Therefore, actinomycin D can be used to investigate whether Ca^{++} mobilization increases Tph2 transcription or, alternatively, increases the stability of Tph2 mRNA molecules. Differentiated RN46A cells were treated with 40 mM KCl for 1 h in the presence of actinomycin D (2.5 μg/ml) or vehicle, and Tph2 mRNA measured relative to GAPDH by quantitative real-time RT-PCR (Figure 3.10). While vehicle-treated cells responded to KCl by increasing Tph2 mRNA levels 48.8 times over control (t-test p=0.007), in actinomycin-treated cells, Tph2 was not stimulated. This result demonstrates that the elevation of Tph2 mRNA level requires de novo transcription. Importantly, actinomycin D alone did not affect Tph2 expression within the 1-h time period.

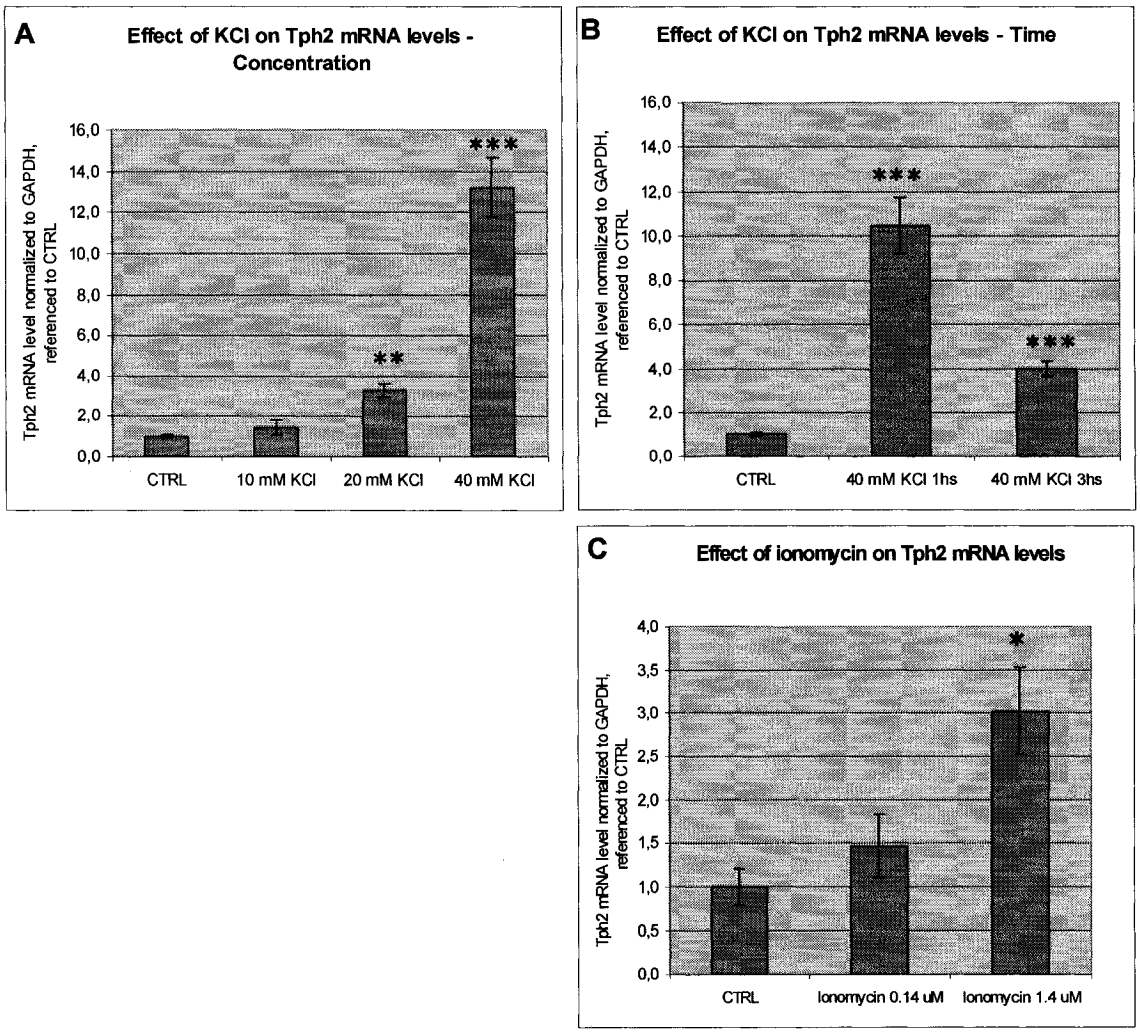

Figure 3.9 - Effect of KCl or ionomycin on Tph2 mRNA levels in differentiated RN46A cells. Tph2 mRNA measured by real-time RT-PCR (SYBR Green chemistry), normalized to GAPDH mRNA and expressed as times of untreated (CTRL). Data shown as mean +/- SEM. **A)** Effect of KCl – concentration dependence. KCl 10 mM (n=3, t-test NS), 20 mM (n=3, t-test p=0.002) and 40 mM (n=3, t-test p=0.001) added 1 h before RNA collection. **B)** Effect of KCl - time dependence. KCl 40 mM added 1 h (n=7, t-test p<0.001) or 3 h (n=7, t-test p<0.001) before RNA collection. **C)** Effect of ionomycin 0.14 (n=3, t-test NS) or 1.4 μM (n=5, t-test p=0.027) added 3 h before RNA collection.

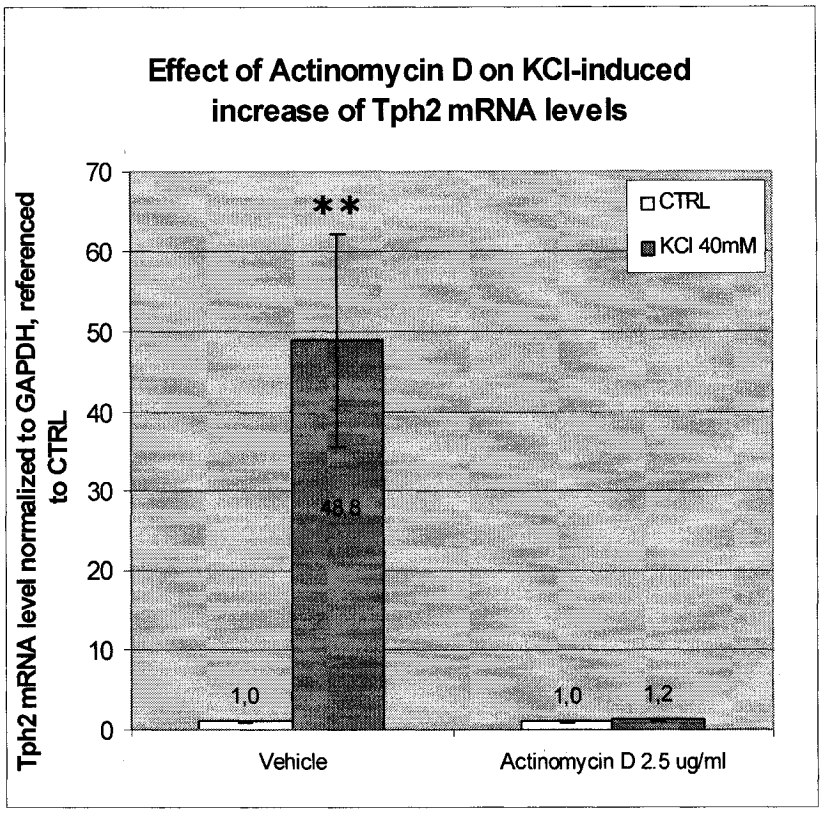

Figure 3.10 - Effect of vehicle (n=6, t-test p=0.007) or actinomycin D 2.5 μg/ml (n=6, t-test NS) on KCl-induced increase of Tph2 mRNA in differentiated RN46A cells. Tph2 mRNA measured by real-time RT-PCR (SYBR Green chemistry), normalized to GAPDH mRNA and expressed as times of CTRL. Data shown as mean +/- SEM.

3.6.3 Effect of Ca^{++} mobilization blockers on KCl-induced increase in Tph2 mRNA levels

In order to investigate the mechanisms that link Ca^{++} mobilization to Tph2 transcription, different treatments were applied to differentiated RN46A cells.

Initially, cells were pre-treated with nifedipine, a dihydropyridine (DHP) that blocks selectively L-type Ca^{++}-channels, for 30 min before the addition of KCl (final concentrations were 1 μM and 10 μM). Voltage-gated L-type Ca^{++} channels open upon depolarization of the membrane, such as that elicited by high K^+ concentration, and allow Ca^{++} ions to enter the cytoplasm. Since RN46A cells express DHP-sensitive (L-type) Ca^{++} channels [Kushwaha and Albert, 2005], it is possible that KCl acted through these channels. However, blocking these channels did not block the K^+-induced increase in Tph2 mRNA level (Figure 3.12). In the presence of vehicle, treatment of the cultures for 1 h with 40 mM KCl resulted in a 7.3-fold increase over control. Similarly, in the presence of nifedipine 1 μM or 10 μM, KCl treatment induced Tph2 expression 7.3 or 9.2 times, respectively, over control. These negative results might imply that the transcriptional effect is attained by a mechanism independent of L-type Ca^{++} channels.

Pre-treatment of the cultures with EGTA (2 mM) also failed to block the K^+-induced increase in Tph2 mRNA (not shown). EGTA chelates extracellular Ca^{++}, substantially decreasing the available ions that can enter the cell after the opening of the channels. The failure of EGTA to prevent the increase suggested that the

Ca^{++} source may be intracellular, or, in any event, that release from Ca^{++} storage sources might be enough to induce Tph2 transcription.

To test this hypothesis, differentiated RN46A cells were pre-treated with BAPTA-AM, an intracellular Ca^{++} chelator. BAPTA-AM enters the cells, chelates Ca^{++} and then is hydrolyzed to BAPTA, a more hydrophilic derivative that cannot cross the plasma membrane again.

When pre-treating cells with BAPTA-AM 10 μM, Tph2 mRNA levels were not significantly different between KCl-treated cultures and controls (Figure 3.12a). On the other hand, vehicle-treated cells responded to KCl by increasing Tph2 mRNA levels 40.5 times over control (t-test $p=0.004$). This result suggests that BAPTA-AM 10 μM blocked the effect exerted by KCl. However it should be mentioned that BAPTA-AM caused an increase in Tph2 mRNA levels even in the absence of KCl. Conversely, a lower concentration of BAPTA-AM (2 μM) did not affect Tph2 expression on its own, but neither did it prevent the KCl-induction conclusively (Figure 3.12b). In this case, KCl significantly elevated Tph2 mRNA levels in vehicle-treated cells 12.9 times over control (t-test $p<0.001$), but it also elevated Tph2 in BAPTA-AM-treated cells 24.2 times over control. Interestingly, this increase over control was not statistically significant (t-test $p=0.064$).

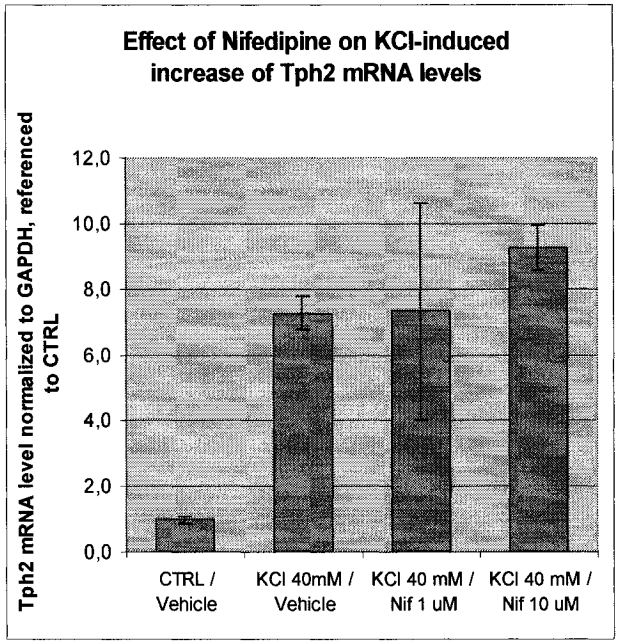

Figure 3.11 - Effect of Nifedipine 1 μM (n=2, t-test NS) or 10 μM (n=3, t-test NS) on KCl-induced increase of Tph2 mRNA in differentiated RN46A cells. Tph2 mRNA measured by real-time RT-PCR (SYBR Green chemistry), normalized to GAPDH mRNA and expressed as times of CTRL. Data shown as mean +/- SEM.

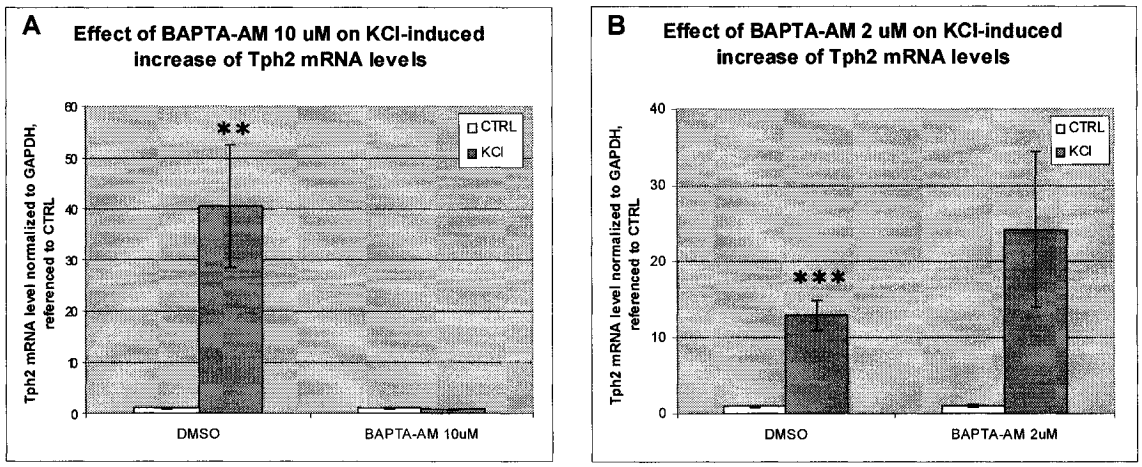

Figure 3.12 - Effect of BAPTA-AM on KCl-induced increase of Tph2 mRNA in differentiated RN46A cells. Tph2 mRNA measured by real-time RT-PCR (SYBR Green chemistry), normalized to GAPDH mRNA and expressed as times of untreated (CTRL). Data shown as mean +/- SEM. **A)** Effect of vehicle (n=3, t-test p=0.004) or BAPTA-AM 10 μM (n=6, t-test NS). **B)** Effect of vehicle (n=4, t-test p=0.001) or BAPTA-AM 2 μM (n=4, t-test NS).

3.7 Endogenous expression of Tph2 in primary neurons

Primary neurons derived from embryonic raphe tissue at E14 were also tested for activity-dependent increase in Tph2 expression. Cultures were treated for 1 h with 40 mM KCl and then Tph2 mRNA was measured by quantitative real-time RT-PCR. This treatment did not induce an increase in Tph2 mRNA in cultures maintained for 6 or 12 DIV (Figure 3.13). Although the cultures are mainly neuronal (glial cells constitute less that 5%), the percentage of Tph2-positive (i.e. serotonergic) cells in these cultures is about 8% [Czesak et al, submitted]. In spite of the relatively low proportion of serotonergic cells in these cultures, any Tph2 mRNA increase should be readily detected by quantitative real time RT-PCR. This negative result contrasts with the response seen in differentiated RN46A cells, another model of serotonergic neuron. A number of hypotheses can be proposed to explain the unresponsiveness of Tph2 gene to KCl treatment in this model.

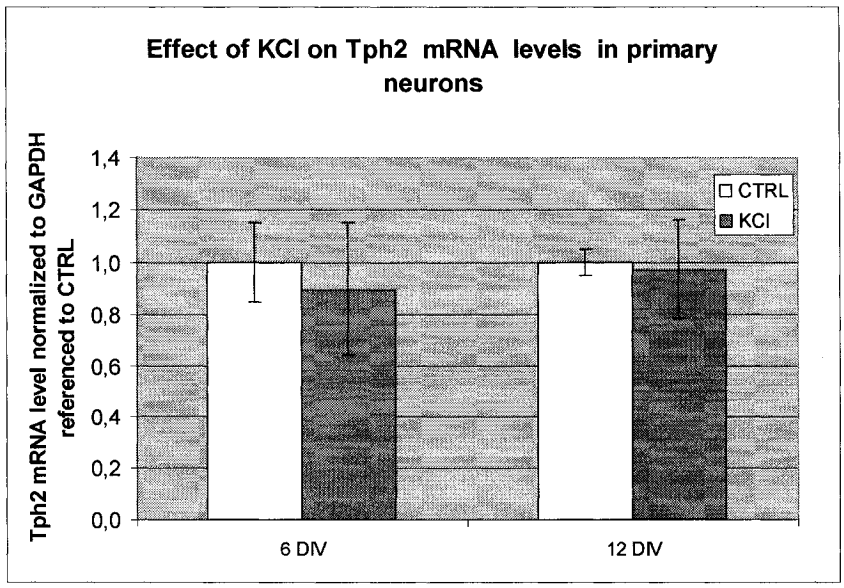

Figure 3.13 - Effect of KCl on Tph2 mRNA levels in raphe primary cultures at 6 (n=6, t-test NS) or 12 DIV (n=6, t-test NS). Tph2 mRNA measured by real-time RT-PCR (TaqMan chemistry), normalized to GAPDH mRNA and expressed as times of CTRL. Data shown as mean +/- SEM.

Table 3.1
Transcription factors identified by Proscan

AP-2	-13
TFIID	-26
AP-2	-122
Sp1	-172
AP-2	-178
CREB	-235
ATF/CREB	-240
E4F1	-240

Table 3.2
Transcription factors identified by TESS

Sp1	-7
TFIID	-26
Pit-1	-30
C/EBPα/β	-45
c-Fos/c-Jun	-130
Sp1	-172
C/EBP	-194
CREB, c-Jun	-235
C/EBP	-290
SRY, TCF-1A	-305
SRY	-320
NF-ATp	-323
SRY	-349
C/EBP	-390
HiNF-A	-390
MEF-2	-396
Pit-1	-401
AP-1, c-Jun	-458
SRF	-481
AP1, c-Jun	-491
GATA-3	-499

CHAPTER IV – DISCUSSION

The serotonergic system is involved in an ever growing number of functions, from autonomic responses to complex behavior. Consequently, dysfunction of brain 5-HT metabolism and neurotransmission is suggested as a contributing cause of numerous mental health disorders. Tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of 5-HT, is the major determinant of the level of 5-HT in the brain and its regulation has the capacity to modulate 5-HT neurotransmission. For decades, TPH was regarded as a single-gene product expressed in the multiple locations of serotonin synthesis. In 2003, Walther et al reported the discovery of a second, neurally-restricted isoform of tryptophan hydroxylase, designated TPH2. Since then, evidence has shown that TPH2 determines brain 5-HT levels and, furthermore, that alteration of its expression level or activity has consequences for 5-HT-related functions and pathologies. For this reason, the study of TPH2 transcriptional regulation is a novel and important area of research in the field of mental health.

While the preponderant role of TPH2 in the brain serotonergic system is well established, to have a comprehensive understanding of its function it is necessary to assess a possible role of TPH1 in the CNS. The almost mutually exclusive expression patterns of the two genes led to the proposal of two serotonergic systems, one central and the other peripheral [Walther and Bader, 2003].

Accordingly, TPH2 would be regarded as the only relevant isoform for the study of serotonin function and pathology in the CNS.

Nevertheless, it cannot be disregarded that TPH1, the so-called peripheral isoform, is also expressed in raphe serotonergic neurons. Low levels of TPH1 mRNA have been detected in rat [Patel et al, 2004], mouse [Kim et al, 2005], macaque [Pecins-Thomsons et al, 1996] and human brainstem [Zill et al, 2005], as well as in cell culture models like rat primary neurons derived from raphe nuclei [Galter and Unsicker, 2000a] and RN46A cells [Siuciak et al, 1998]. In all cases, Tph1 quantities have been minute, but always readily detectable by RT-PCR. More importantly, Tph1 mRNA expression in the rat and mouse raphe follows a temporal pattern of low embryonic expression, peaking around post-natal day 21 (P21) and declining back to barely detectable levels during adulthood [Rind et al, 2000; Nakamura et al, 2005]. Nakamura et al also claim that Tph1 enzyme has higher affinity than Tph2 for tryptophan in conditions that resemble the developing raphe [Nakamura et al, 2005]. In addition, SNP association studies have repeatedly linked TPH1 to suicidal behavior [Bellivier et al, 2004] and depression [Gizatullin et al, 2006]. This line of evidence suggests a role for TPH1 in the developing serotonergic system and also that the two-TPH two-system model may be over-simplistic.

On the other hand, TPH2 is overwhelmingly more abundant than TPH1 in the adult brain (of any species so far studied) and, importantly, also in the mouse raphe nuclei at P21, during the peak of Tph1 expression [Nakamura et al, 2005]. What is more, it has been shown experimentally that Tph2 controls brain 5-HT level in the adult mouse [Zhang et al, 2004]. Finally, if Tph1 played a role in

development, it would be expected that its disruption caused long-term behavioral consequences. In other occasions, disruption of serotonergic neurotransmission during development or early life, such as in 5-HT1A KO mice or early-life SSRI administration, has led to a variety of irregular behavioral responses [Ansorge et al, 2004; Gross and Hen, 2004]. Conversely, Tph1 KO mice responded normally to behavioural tests, although these only tested anxiety-like behavior and locomotor activity [Walther et al, 2003].

In summary, TPH2 remains as the most relevant isoform in the CNS serotonergic system, yet a role for TPH1 cannot be entirely discarded.

4.1 Analysis of results

The study of promoters and transcriptional regulatory mechanisms requires the precise identification of the transcription start site (TSS). In the case of the TPH2 gene, the reported TSS was suggested from the original cloning of the full length cDNA by Walther and coworkers [Walther et al, 2003]. Importantly, these data were obtained by 5'-RACE of RNA extracted from Tph1 KO mouse brain. Subsequently, based on the mouse sequence, the human homolog was cloned and a single, conserved TSS was reported. There is no indication in this report of a screening to identify alternative TSS. Since the use of multiple promoters is a common mechanism of transcriptional regulation, it was necessary to confirm that the reported site was indeed the only TSS for the human TPH2 gene in order to localize relevant promoter elements.

The results obtained from the 5'-RACE experiment indicated that splicing variants originating from upstream TSS are unlikely. Firstly, the 5'-RACE PCR yielded only one product, while alternative TSS would most likely result in multiple products. Secondly, none of the cloned products presented sequences that did not belong to the known 5-UTR or CDS. On the other hand, it is possible for an alternative TSS to have gone undetected if a) a variant spliced out exon 4, eliminating the annealing site of the TPH2-specific primer or b) a variant started so far upstream that either the polymerases in the reverse transcription or in the 5'-RACE failed to complete the product [Matz et al, 2003].

Since a whole-brain adult human cDNA library was used as a template, these results are valid only in such a context. Active TSS might change depending on the tissue or stage of development, so the existence of different alternative TSS active earlier in development or in other TPH2-expressing cell types, like enterochormaffin neurons, cannot be ruled out.

TPH2 has been cloned from a number of species and yet no alternative TSS has been reported, except a single clone from human amygdala picked up by "Full-length long Japan" sequencing consortium [Ota et al, 2004]. This transcript, which overlaps the first five exons of TPH2, starts 131 bp downstream of the more common TSS. The significance of this finding is not clear, and may just represent an incomplete TPH2 transcript.

To sum up, the 5'-RACE performed on an adult brain library identified a single TSS, indicating that the transcriptional regulation of the human TPH2 gene is controlled by only one promoter, which would likely be in the 5' flanking region of the gene.

The transcriptional activity of the DNA upstream of the TSS was evaluated by means of reporter gene assays of deletion constructs. The results indicated that the region could drive transcription of the reporter gene significantly more than an empty control vector. In other words, the TPH2 promoter is located in the proximal region upstream of the TSS, both in the human and rat genes. What is more, the rat promoter cloned in the antisense orientation inhibited the transcriptional activity of the vector, demonstrating orientation-dependence that is typical of a promoter rather than an enhancer, which is generally orientation-independent.

The activity of the promoter was similar in all cell lines tested, independently of endogenous TPH2 expression, indicating lack of cell-type specificity in the minimal TPH2 promoter region. Regulatory element conferring cell-type specificity to transcription may lay outside of the 1.4 kb DNA fragment covered by these constructs. Moreover, a longer human TPH2 promoter construct (4.9 kb) tested in HEK 293 and differentiated RN46A cells rendered similar results (data not shown), further extending the non-specific DNA region to 4.9 kb upstream of the TSS. Similarly, the rat -1004 construct did not show any cell-type specificity either.

Tissue-specificity can prove difficult to characterize using cell lines, as exemplified by the study of the rat TH promoter. In this case, a 272-bp-long promoter construct was enough to drive cell-specific transcription of a reporter gene [Cambi et al, 1989], but at least 4.5 kb were necessary to obtain tissue-specific expression in transgenic mice [Schimmel et al, 1999]. Accordingly, neuronal promoters transfected into non-neuronal cells are often active. In

principle, it can be assumed that the elements that confer cell-type specificity are located further upstream or downstream of the transfected stretch of DNA. Nevertheless, there are reasons to believe that transient transfection of plasmids may not completely reflect the regulatory events that take place on the genomic DNA.

To begin with, although plasmids in the nucleus could form nucleosomes, they do not interact with histones like the genomic DNA does, hence incompletely modeling the role of chromatin-remodelling proteins [Belyaev et al, 2004]. In particular, Belyaev and coworkers made the case that the same RE-1 sequence recruited distinct transcriptional regulatory protein complexes when it acted as an endogenous versus as a transiently-transfected target. Secondly, tissue-specific repression of genomic DNA can be achieved by DNA methylation or diverse post-translational histone modifications, while the transfected plasmid enters the cell unmethylated and will bind unmodified histones, without the epigenetic regulation present in endogenous genes of the host cell. Finally, some genes are located in a chromosome so that insulator regions or chromatin boundaries protect them from the activating effect of enhancers [West and Fraser, 2005], a protection that is likely lost for transfected promoters.

Due to these caveats of the reporter gene assays to define tissue-specific elements, it might still be possible that elements conferring tissue-specificity reside in the first 1.4 kb of the human TPH2 promoter. Investigation to find these elements should consider that tissue-specific gene expression of TPH2 must be tightly related to the differentiation of serotonergic neurons during development. In

principle, TPH2 expression requires factors for neuronal restriction and specific activators that might be shared with other serotonergic genes. A number of transcription factors are known to be required for the development of a serotonergic phenotype, such as Nkx2.2, Lmx1b, and Pet-1 [Cheng et al, 2003]. Of these, the Ets-domain transcription factor Pet-1 is specifically expressed in serotonergic neurons and potentially holds the key to serotonergic gene expression. Experimentally-confirmed Pet-1 binding sites have been found in the 5' flanking region of 5-HT1A, 5-HTT, TPH1 and AAAD genes [Hendricks et al, 1999], and its disruption caused substantial depletion of TPH and other serotonergic protein markers [Hendricks et al, 2003]. It is not known yet if Pet-1 binds and regulates the human TPH2 promoter, although manual inspection of the sequence detected two putative sites located at positions -554 and -890. The activity of the constructs -503, -683 and -1082 was not significantly different in any of the cell lines, suggesting that these sites are not active as enhancers in this context. Alternatively, since Pet-1 is aberrantly expressed in various cell lines it may activate the promoter in these reporter constructs even though the endogenous gene remains silenced. However, transfections of PET-1 alone are not sufficient to induce expression of Ets-containing reporters, suggesting that additional factors are required.

Based on the reporter gene assays in all three cell lines, the analysis of the first 1.4 kb revealed the presence of a repressor element somewhere between -88 and -179 bp from the TSS. There is a possibility that this repressing activity derived

from the reported RE-1-like element located at position -113 [Mueller and Patel, 2005], but it is also possible that it was consequence of still unknown elements, or even additional ones. To test the repressor activity, Mueller and Patel isolated the RE-1-like element and cloned it into a reporter promoter. Since repressor activity of RE-1-like sequences is dependent on the promoter context [Mieda et al, 1997; Myers et al, 1998], its activity within the TPH2 promoter is yet to be evaluated. Further studies to resolve the involvement of REST may include measuring the activity of constructs lacking the RE-1-like element (due to deletion or mutation of core nucleotides) and ChIP assays to investigate endogenous binding.

It remains a question why the TPH2 promoter was under the effect of a repressor in Tph2-positive cells, like differentiated RN46A cells. In spite of being a Tph2-expressing model of serotonergic neurons, the cells never drove high expression levels of Tph2 mRNA or protein. In the undifferentiated state, RN46A cells proliferate, have a fibroblast-like morphology and express functional REST [Lemonde et al, 2004]. The differentiation process triggers the development of both the neuronal and the serotonergic phenotype of the cells [White et al, 1994; Eaton et al, 1995], but it is not known if they continue expressing REST or any other factor that could be preventing Tph2 transcription. There are examples where REST acts during neuronal differentiation as a modulator rather than as a silencer, like the case of the glutamatergic AMPA receptor subunit GluR2 [Myers et al, 1998]. In addition, REST repression of the GluR2 promoter during differentiation can be reversed by BDNF treatment [Brene et al, 2000], a neurotrophin that increases TPH expression in differentiating RN46A cells and primary raphe

neurons as well [Eaton et al, 1995; Galter and Unsicker, 2000a]. Nonetheless, even if REST regulates the neuron-specific expression of TPH2, an additional mechanism is necessary to explain how its expression is restricted to 5-HT neurons.

While differentiated RN46A cells express only low amounts of Thp2, serotonergic neurons in rat raphe primary cultures demonstrate high Tph2 mRNA expression. However, transcriptional activity of the human -278 and the rat -1004 constructs was very similar to that observed for differentiated RN46A cells. Primary cultures used for transfection were derived from the rostral rhombencephalon of rat E14 embryos and cultured for 12 days. Although the cultures are mainly neuronal (glial cells constitute less that 5%), the percentage of Tph2-positive (i.e. serotonergic) cells in these cultures is about 8% [Czesak et al, submitted]. Thus, in practical terms, for transfections the raphe culture is composed primarily of non-serotonergic cells, in agreement in the low activity levels obtained in the reporter gene assays. The observed activity may reflect the low activity in 92% of the transfected non-serotonergic cells and high activity from the serotonergic 8%, hence diluting any effect. To clarify if the TPH2 promoter is distinctly active in serotonergic cells, it would be necessary to follow its transcriptional activity in identified serotonergic neurons. This could be achieved by transfecting the cultures with constructs where the TPH2 promoter is cloned before a vector carrying a marker protein such as GFP as reporter gene, to later evaluate the level of marker expression by fluorescence or immunocytochemistry only in cells immunopositive for 5-HT or TPH.

The pattern of transcriptional activity for longer constructs was not uniform across cell types, unlike the case of the two shortest ones. Transcriptional activity in HEK 293 cells, of human origin, remained repressed in all the larger constructs. On the contrary, activity in the two rat cell lines augmented from the -179 to the -278 and again from the -278 to the -503 promoter construct. Analysis of the sequence showed a consensus Sp1 site starting at position -172 that is disrupted in the -179 promoter construct, suggesting a candidate transcription factor to explain the gain in activity by the -278 construct. The Sp1 transcription factor is an activator of basal transcription that acts on promoters of a numerous and diverse set of genes [Li et al, 2004b]. Besides electrophoretic mobility shift assays and reporter gene assays of mutated constructs, Sp1 involvement can be investigated using mithramycin A, an inhibitor of Sp1-DNA interaction.

The results from the reporter gene assays predict another enhancer element capable of stimulating basal transcription to be between positions -278 and -503. Analysis of the 5' flanking region of the human TPH2 genes using the TESS software identified numerous putative elements that will require experimental confirmation of functionality. The search of the TRANSFAC database serves as a first approach to the identification of the TFBSs, either enhancers in the distal promoter or other elements closer to the TSS. However, the significance of computer-based searches of TFBSs is relative due to the large amount of false positive results (i.e. sequence hits without functionality), so much that it has been estimated that, in average, only one every thousand hits is functional [Wasserman

and Sandelin, 2004]. Raising the detection threshold might miss important functional elements, given the relatively weak consensus sequence requirements of many transcription factors for DNA binding. In fact, a genome-wide study of highly-degenerate versions of known TFBSs, including RE-1 and CRE, found a non-random distribution throughout the genome that parallels that of less degenerate sequences, suggesting a function for these sites as well [Zhang et al, 2006].

TFBSs are subject to evolutionary constraints, although not as strongly as coding sequences [Dermitzakis and Clark, 2002]. Therefore, identification of conserved regulatory elements might be more meaningful than the analysis of a single sequence. However, when 2000 bp of the rat and human 5' flanking regions were analyzed with the zPicture plus rVista 2.0 software [http://zpicure.dcode.org; http://rvista.decode.org], no element was found to be 100% conserved and aligned in the two sequences. Therefore, it becomes difficult to speculate about the identity of any enhancer located in the distal promoter and more experimental screening is required.

Independently of the cell line that hosted the promoter constructs, their overall transcriptional activity was low. In particular, the minimal promoter (-88) averaged an activity of 2.5 to 3.0 times the control. Activities these low are not unheard of in the published literature, including, for instance, promoters of the human DA transporter [Sachetti et al, 1999], murine 5-HTT [Sakai et al, 2003], rat 5-HT1A receptor [Storring et al, 1999], and human TH [Kim et al, 2003]. This

response could be originated due to the presence of another repressor element between position -88 and the core promoter (TATA box goes from -31 to -26). A TESS search for putative TFBS in this stretch revealed two elements scoring high, a pituitary specific transcription factor-1 (Pit-1) site at -30 bp and a CCAAT/enhancer binding protein α (C/EBPα) site at -45 bp. Pit-1, a POU domain transcription factor expressed only in the pituitary gland, is mainly an activator, although Pit-1 can function as a repressor too [reviewed in Andersen and Rosenfeld, 2001]. C/EBPα, a bZIP transcription factor that can bind CRE sites, is also known as an activator [reviewed in Ramji and Foka, 2002]. These are unlikely candidates to be repressors in serotonergic neurons, and the existence of either a novel regulatory element or a highly-degenerate, but functional, TFBS cannot be discarded.

Alternatively, it could be speculated that low basal promoter activity resulted from the design of the deletion constructs. The genomic DNA inserts stretch from the distal end to position +1 of the TSS, while it is known that core promoter elements can be located as far as 40 bp downstream of the TSS [Smale and Kadonaga, 2003]. These elements include the initiator sequence (Inr), which goes up to position +4, and at least two elements further downstream, the motif 10 element (MTE) and the downstream promoter element (DPE) [Butler and Kadonaga, 2002]. These elements are relevant for basal and also specific transcription of a number of genes, but some evidence suggests this is not the case for TPH2. Firstly, when transfected into HEK 293 cells, a reporter construct prepared by fusing the -278 promoter sequence upstream of the full-length 5'-UTR

did not show higher luciferase activity than the original -278 construct lacking 5'-UTR sequence (data not shown). Furthermore, manual inspection of the human TPH2 promoter did not detect consensus Inr or MTE sequences, and the DPE element is observed mostly in TATA-less promoters [Butler and Kadonaga, 2002]. In all, rather than having an incomplete core promoter, it seems more likely that the -88 TPH2 promoter construct presents low intrinsic activity. This might be due to the presence of an unidentified repressor or the absence of the appropriate activators in the cell lines used.

Taken together, these data from reporter gene assays suggest a complex combination of regulatory factors acting on the 5' flanking region of the TPH2 gene. In particular, it can be concluded that at least one repressor element lies in the first 179 bp of the promoter and that one or more activating elements are located further upstream.

Conclusions about tissue-specificity of expression are more the subject of speculation. As a hypothesis, expression of serotonergic genes, notably TPH2, might require very specific activators, which would appear in serotonergic neurons during development (for instance, transcription factor Pet-1 would be a candidate). These activators, expected to be present in differentiated RN46A cells (hence the endogenous expression there), did not show any effect, implying that a) the binding site lies somewhere outside the 1.4 kb sequence tested or b) the mechanism of activation cannot take place in transiently-transfected plasmids. The data collected

in these experiments serve as a first step towards the finding of those, for now, hypothetical factors.

The results from the reporter gene assays can also contribute to clarify other questions. A number of polymorphisms have been studied in the 5' flanking region of the human TPH2 gene. Of particular interest is SNP rs4570625, at position -703, because the T allele has been associated to increased amygdala neural activity by two groups independently [Brown et al, 2005; Canli et al, 2005]. Since augmented amygdala reactivity has been also associated to the short allele of the 5-HTT promoter polymorphism (i.e. less 5-HTT, more extracellular 5-HT), the authors proposed that the T allele might be associated to increased TPH2 expression. The T allele, as opposed to the G, is the less frequent one, being present in 22% of the chromosomes. However, it is the allele present in the promoter constructs used in this study. Given its location in a regulatory region, this SNP is candidate to be functional. In fact, it is located only 7 bp downstream of a site that potentially binds the POU domain transcription factor Oct-1. On the other hand, the transcriptional activities of the constructs -683 (without the Oct-1 site) and -1082 (including the site) were not significantly different in any of the cell lines. The final answer about the functionality of this SNP will include direct comparison of transcriptional activities of each allele in reporter gene assays and also comparison of DNA-protein binding ability.

The number of neuronal genes regulated by activity-dependent Ca^{++} influx is well into the hundreds [Li et al, 2004a]. The similarity between regulatory pathways of TPH2 and TH, a Ca^{++}-regulated gene, as well as the nature of the cues regulating TPH expression prompted the hypothesis that TPH2 might belong to the group of activity-dependent genes. Indeed, experimental results seem to indicate so. Both the human and rat TPH2 promoters were activated by Ca^{++} mobilization in differentiated RN46A cells, a neuronal cell line. When the cells were transfected with the empty vector, Ca^{++} mobilization did not have any effect on luciferase activity. On the other hand, cells transfected with the TPH2 promoter increased luciferase activity by an average 60%. Since this response was observed in all the constructs, it can be assumed that the Ca^{++}-sensitive element lies in the first 88 bp upstream of the TSS.

TESS screening detected several Ca^{++}-responsive elements in the proximal human TPH2 promoter, including a CRE site at position -235 and a c-Fos / c-Jun site at -130. In addition, within the first 88 bp, there is a C/EBPα/β site spanning from position -45 to -54 upstream of the TSS. Members of the C/EBP family are inducible transcription factors, responding to NGF, cAMP or adrenergic stimulation [Menard et al, 2002; Seitz McCauslin et al, 2006], but the response to neuronal activity is not clear. Nonetheless, since C/EBP and CREB transcription factors belong to the bZIP family, it is possible for CREB to bind non consensus C/EBP sites [Flammer et al, 2006]. Like other bZIP transcription factor binding sites, C/EBP and CRE are composed of two half sites in an inverted pair, separated by two pyrimidines. In particular, CRE consensus site is ATGA-CG-TCAT, whereas

C/EBPβ consensus site is ATTG-CG-CAAT [Ramji and Foka, 2002]. Interestingly, chimeric C/EBPβ-CRE binding sites have been reported to bind CREB functionally. As reviewed by Flammer and colleagues, chimeric sites have been shown to regulate transcription of the hepatitis B virus X protein gene, proprotachykinin-1 gene and the serotonin N-acetyltransferase gene [Flammer et al, 2006]. The human TPH2 promoter C/EBPα/β element detected by TESS can be seen as a chimeric C/EBPβ-CRE site that matches all eight nucleotides of the inverted pairs to those of the serotonin N-acetyltransferase gene. This raises the interesting possibility that Ca^{++}-responsiveness is conferred by CREB interaction with the chimeric element. Since, at least in vitro, CREB and C/EPBβ homodimers bind the chimeric site with similar affinities [Flammer et al, 2006], transcription can be in part regulated by competition and availability of this factors.

This element is not fully conserved in the rat promoter, which was also sensitive to Ca^{++} stimulation in transfected cells. Since the response to ionomycin induction was tested in a 1004-bp promoter construct, it is likely that additional Ca^{++}-sensitive sites were present. Similarly, the functionality of putative activity-dependent elements further upstream in the human promoter cannot be discarded, even when the shortest promoter construct responded as much as the -1402 construct. These elements might not have additive effects or may just be active in different conditions, as observed for the CRE and AP-1 elements in the TH promoter [Ghee et al, 1997].

Importantly, induction of TPH2 promoter activity was only seen in differentiated RN46A cells (Tph2-positive), as opposed to non neuronal cell lines L6 (Tph2-negative) or GH4C1 (Tph2-positive). This result prompts the hypothesis that at least one neuronally-restricted, Ca^{++}-dependent factor participates in the regulation of Tph2 gene expression. This factor could be a DNA-binding protein, a cofactor, a component of the signaling cascade or even a yet unknown small non coding RNA with regulatory functions.

The idea of a cell-type specific transcription factor and the hypothesis of CREB involvement are not contradictory with each other. Given the combinatorial nature of gene expression regulation by transcription factors, most likely multiple proteins are cooperating to confer Ca^{++} responsiveness to the TPH2 promoter. An example of this is the case of the BDNF promoter III, where CREB requires the presence of cell-type-specific factor CaRF to induce transcription after neuronal activity [Tao et al, 1998; Tao et al, 2002].

Future investigation of the Ca^{++} responsiveness of the TPH2 promoter should start by confirming the activity of candidate elements. This could be done by means of reporter gene assays using constructs where the elements are deleted or mutated. A second issue is whether this response is specific of serotonergic cells or neurons in general, so the TPH2 promoter should be tested in neuronal, non serotonergic cells. Candidate model systems for these experiments are PC12 cells or primary neurons from any source other than midbrain. A response specific of serotonergic neurons would not only be a great advance in the understanding of

serotonergic differentiation but also a promising tool to design therapies that target only the serotonergic system.

In summary, the investigation of the human TPH2 promoter presented in this work permits the construction of a first sequence map based purely in experimental results (Figure 4.1). The model postulates a Ca^{++}-sensitive element in the proximal 88 bp, near a consensus TATA box, that is exclusive to neurons. In addition, there is at least one repressor element within 179 bp of the TSS. Further upstream, there are at least two enhancer elements before reaching the 500 bp mark.

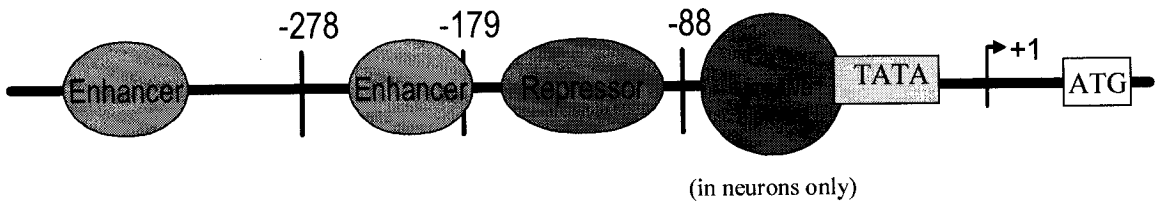

Figure 4.1 – Schematic diagram showing the human TPH2 promoter. All features included are derived from experimental results only.

Quantitative real time RT-PCR was used to test whether the Tph2 promoter in its genomic context is also Ca^{++}-responsive. This time, besides ionomycin, high KCl concentration was used as a means to mobilize Ca^{++} into the cells. At high concentrations, ionomycin is a more powerful Ca^{++} mobilizer but is toxic. High KCl concentration has the advantage of being non-toxic and more physiological, since Ca^{++} influx occurs through Ca^{++} channels resembling synaptic activation. In any event, both treatments substantially elevated Tph2 mRNA levels in a short period of time.

The steady-state concentration of mRNA depends on the rate of decay as much as it depends on the rate of transcription. Therefore, it is possible that Ca^{++} mobilization acted by decreasing the degradation of Tph2 mRNA instead of increasing its transcription. Since actinomycin D blocked the KCl-induced increase in Tph2 mRNA, it can be concluded that KCl triggers an increase in transcription. However, altered mRNA stability may still play a role in regulation of Tph2 gene expression. A recent report by Chen et al showed functional polymorphisms in the 3'-UTR of the macaque TPH2 that correlated with HPA axis activity [Chen et al, 2006]. According to this model, the 3'-UTR alleles that conferred higher in vitro expression of a reporter gene were associated with lower HPA axis response.

Further information about the mechanism of induction of transcription could be gathered by blocking translation. Addition of cycloheximide, a blocker of translation, prior to the Ca^{++} mobilization could be used to determine whether

protein synthesis is required for KCl-mediated induction. Activity-dependent induction of some genes requires protein synthesis of IEG, like Fos and Jun. On the other hand, activation of certain genes, like BDNF from promoter III [Tao et al, 1998] or proenkephalin [Hahm et al, 2003], is independent of protein synthesis, since it is induced by post-translational modification of transcription factors, primarily CREB phosphorylation. Both CRE and AP1 binding sites are found in the human TPH2 promoter, so in principle, any of these mechanisms may apply.

Preliminary attempts to block extracellular Ca^{++} to enter the cell did not succeed in preventing the KCl-induced increase in Tph2 mRNA. Nifedipine blocks DHP-sensitive channels only, so Ca^{++} can enter the cell through other ways, such as N- or P/Q-type VGCCs or NMDA receptor. Together with the NMDA receptor, the DHP-sensitive L-type VGCC has been repeatedly associated to activity-dependent regulation of transcription [West et al, 2002]. Intriguingly, addition of 2 mM EGTA did not prevent the increase in Tph2 mRNA levels either, suggesting that intracellular Ca^{++} may be involved. Minute amounts of Ca^{++} can enter the cell and trigger CICR from intracellular stores. To test this hypothesis, intracellular and not extracellular Ca^{++} should be sequestered.

The intracellular Ca^{++} chelator BAPTA-AM blocked the KCl-induced effect, since Tph2 mRNA levels after KCl treatment are comparable to those of untreated cells. However, when the cells were treated with BAPTA-AM 10 μM alone, Tph2 mRNA levels resulted substantially higher than in the DMSO-only controls, an

effect similar in magnitude to that of KCl. So it is possible that KCl did not induce transcription in the BAPTA-AM-treated cell simply because the induction had already taken place. The mechanisms that may trigger such a response are not clear. Chelation of intracellular Ca^{++} has severe consequences for the cell. Primary neurons loaded with BAPTA-AM showed signs of apoptosis as early as 4 h after the treatment and necrosis 24 h later [Han et al, 2001]. What is more, loading of primary neurons with BAPTA-AM induced endoplasmic reticulum dysfunction, probably by chelating luminal Ca^{++} [Paschen et al, 2003]. ER dysfunction, in turn, can alter gene expression at the level of transcription [Kaufman, 1999]. In summary, it is possible that BAPTA-AM rapidly triggers metabolic pathways with unexpected consequences, which may alter Tph2 gene expression. In addition, the hydrolysis of BAPTA-AM may generate toxic products that generate a stress response to induce TPH2 expression.

In summary, Ca^{++}-mobilizing agents rapidly increased the endogenous transcription of the Tph2 gene in differentiated RN46A cells. The mechanism remains unclear, although most likely it involves Ca^{++}-mediated transcription.

Involvement of extracellular Ca^{++} or L-type VGCC cannot be ruled out for now, since it is possible that the KCl effect might be prevented by varying the experimental conditions in the strategies already tested. In addition, new experiments to elucidate the pathway involved could include mobilization of Ca^{++} from internal storage or blocking of nuclear kinases, particularly CaMK II.

In rat raphe primary neurons, KCl did not induce Tph2 mRNA expression. There are a number of hypotheses to explain the lack of responsiveness to KCl in the primary cultures. To begin with, the response kinetics in primary cultures may be different than that of differentiated RN46A cells. In this case, 1 h might be insufficient time and longer treatments might be required. Varying times reflect different mechanisms regulating gene transcription. For instance, in cortical neurons, treatment with 50 mM KCl triggers increase of c-Fos mRNA levels in 5 min to peak in 1 h, whereas BDNF levels only rise after 30 min of stimulation to reach a maximum after 3 h [Tao et al, 1998]. Both genes are activated by CREB, but while the c-fos promoter is ready, BDNF promoter III is occupied by MeCP2, a repressor that requires phosphorylation to abandon the DNA and allow the activators to bind [Chen et al, 2003b]. Thus, it can be hypothesized that serotonergic neurons in culture regulate activity-dependent Tph2 transcription with additional layers of complexity that may require more time (or stimulation of a different nature) than observed in the RN46A cell line.

It is also possible that the presence of various non-serotonergic cell types in the culture (GABAergic, dopaminergic, glial cells) affect the ability of the serotonergic neurons to respond. While serotonergic neurons comprise about 8% of the population, GABAergic neurons account for more than 50% [Czesak et al, submitted]. KCl depolarization may induce a GABAergic discharge in the culture, actually inhibiting neuronal activity of the surrounding cell types. In the DRN in vivo, GABAergic neurons input on the serotonergic neurons, modulating the activity of their ascending projections. This interaction is achieved through ionotropic $GABA_A$

receptors as well as G protein-coupled $GABA_B$ receptors [Innis and Aghajanian, 1987].

Alternatively, it cannot be ruled out that neurons in primary cultures might be too immature. Although Lautenschlager et al reported that raphe cultures develop serotonergic characteristics (5-HT synthesis, release and uptake) after 9 DIV [Lautenschlager et al, 2000], it is still possible that they do not express a fully differentiated phenotype, or even the adequate Ca^{++} channels or transcription factors for this kind of response. Activating the cells with ionomycin instead of KCl would be an alternative to bypass the absence of Ca^{++} channels. On the other hand, presence of active Ca^{++} channels in serotonergic cultured neurons could be checked electrophysiologically.

Finally, while these cultures were obtained from rostral raphe nuclei, the RN46A cells were derived from medullary raphe. Serotonergic neurons do not belong to a uniform population and their activity is differentially regulated in different regions. For instance, recent studies reported that Tph2 mRNA levels do not respond equally in distinct raphe nuclei to ovarian hormones [Hiroi et al, 2006]. Of particular importance are the differences in transcription factors. Lmx1b mutant mice do not have problems in medulla serotonergic neurons [Cheng et al, 2003], while GATA-3 disruption led to defects only in caudal serotonergic neurons, but not rostral [van Doorninck et al, 1999]. Finding specific differences between these two subgroups of serotonergic neurons represents a contribution to the design of therapies with smaller side effects and very precise purposes.

4.2 Future directions

TPH2 occupies a fundamental role in the CNS serotonergic system and for this reason it is at the same time a candidate contributing cause to a number of psychiatric and non psychiatric maladies and a potential target of therapies. Future directions of investigation on TPH2 should address the issue of turning it into a tool to mitigate disease.

Research on the transcriptional regulation of TPH2 should continue to study the pathways linking extracellular signal to gene expression. For example, study of the relationship between 5-HT autoreceptor activation, TPH2 expression and the effect of SSRIs, in order to improve efficacy of these medications.

Alternatively, study of TPH2 could focus on TPH2 as a target. Given the number of processes in which it is involved, aiming at TPH2 as a target directly might have broad effects and unexpected consequences. Alternatively, aiming at influencing the factors that differentially regulate its expression under a particular circumstance (for instance stress or drug consumption) or a distinct subpopulation of serotonergic neurons seems to be a wiser approach.

Targeting of nuclear factors is not utopist. Although membrane proteins, like G protein coupled receptors and monoamine transporters are the most common target of medications, alternative strategies exist to reach to nuclear proteins. Protein translocation domains (PTD) are peptide sequences that can transport a macromolecule into the cell and then into the nucleus, so transcription factors can become direct target of medication. Some of the proteins that carry a PTD and are

being tested include Drosophila homeobox protein Antennapedia, the HIV-1 transcriptional factor TAT and VP22 from HSV-1 [Naguchi and Matsumoto, 2006]. These domains can be tagged to entire proteins and delivered in vivo, like the case of PTD-TH, which crosses the BBB and goes into the striatum carrying TH, as a potential medication against Parkinson's disease [Wu et al, 2006]. In terms of transcription factors, for instance, a protease-resistant peptide that prevents increase in c-Jun activation and c-Fos transcription is being investigated as a neuroprotective agent for stroke [Borsello et al, 2003].

The search for targets of this kind of approach, to be used in a particular subpopulation of serotonergic neurons, in order to modify TPH2 expression and alter 5-HT neurotransmission with a specific objective, might be a goal worth aiming at in the future investigation of TPH2 gene regulation.

4.3 Conclusions

To sum up, the work presented here supports the following conclusions:

a) The reported transcriptional start site for the human TPH2 gene has been experimentally confirmed.

b) The human TPH2 5' flanking region was cloned and found to be transcriptionally active. This promoter region was analyzed uncovering the presence of a core promoter (downstream of -88), a repressing element

somewhere between -179 and -88, and an activating element located upstream of -278, but downstream of -503.

c) The human TPH2 promoter can be activated by Ca^{++} mobilization in a serotonergic, neuronal cell line, but not in other cell lines. The element responsible of this effect is located in the proximal promoter region (-88 and downstream).

d) The 5' flanking region of the rat Tph2 gene is transcriptionally active and has Ca^{++}-responsiveness characteristics similar to the human promoter.

e) Endogenous transcription of Tph2 can also be induced by Ca^{++} mobilization in a serotonergic, neuronal cell line. This induction can potentially be blocked by intracellular Ca^{++} chelators, but not by L-type Ca^{++} channel blocking or EGTA, indicating the possible intracellular origin of the Ca^{++} increase that triggers Tph2 expression.

This work is the first one to identify the promoter region of the human TPH2 gene and to report the activity-dependent regulation of transcription. The importance of Ca^{++}-regulated gene expression is underscored by increasing evidence linking neuronal activity to gene regulation, thus establishing a mechanism for long-term neuronal changes. Tryptophan hydroxylase-2 lies in the center of the serotonergic system and controls the levels of 5-HT in the brain, therefore being likely associated to modulation of mood and behavior in health and disease. Understanding its transcriptional regulation is necessary to fully

comprehend its role in the mechanisms of mental disease and to devise new, specific therapeutic strategies in the future.

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