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An investigation of the link between the bacterial derived queuine molecule and tyrosine production

By

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Declaration

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Abstract

Queuine, a dietary derived 7-deazaguanine molecule, has previously been shown to prevent lethality and other symptoms in germ-free mice that were maintained on a tyrosine-free diet. This work suggested a link between queuine and tyrosine biosynthesis or tyrosine utilisation in higher eukaryotes.

In this report, it is demonstrated that tyrosine biosynthesis is impaired in HepG2 cells grown under queuine-deficient cell culture conditions after 30 minute incubation with increasing phenylalanine concentrations. To further analyse this phenomenon, tyrosine biosynthesis was also analysed in TGT gene-trap mice. In vivo, the TGT protein functions in conjunction with a mitochondrial bound protein, known as Qv1, to insert queuine into transfer RNA. It was observed that TGT-gene trap mice have a severely compromised ability to synthesise tyrosine over time from exogenously administered phenylalanine. The decrease in tyrosine synthesis in queuine deficient HepG2 cells and TGT gene-trap mice was not due to changes in the activity or expression of the phenylalanine hydroxylase enzyme (a liver and kidney specific monooxygenase essential for phenylalanine to tyrosine conversion) or the expression of tyrosine hydroxylase in the brain, a protein involved in the metabolism of tyrosine. Changes were observed in phenylalanine hydroxylase cofactor, tetrahydrobiopterin and in particular the accumulation of its oxidised metabolite 7,8-dihydrobiopterin, in liver, urine and plasma.

Tetrahydrobiopterin is an essential cofactor for the synthesis of key neurotransmitters such as dopamine, adrenaline, nor-adrenaline, serotonin and nitric oxide through the activity of the aromatic amino acid hydroxylase enzymes and the enzyme nitric oxide synthase. An examination of various brain sections of TGT gene-trap mice showed variable levels of biogenic amines, in particular dopamine and serotonin metabolites. Further research into the behavioural characteristics of TGT genetrapped mice led to the discovery that only female mice seemed to be affected and displayed lower anxiety levels and higher exploration activity.

Research into the biochemical composition of plasma, liver and brain sample led to observation that the absence of queuosine-tRNA results in decreased levels of lactate, alanine and creatine. As these are signature molecules of mitochondrial function it

appears queuosine deficient mice are relying on oxidative phosphorylation for energy to a greater extent than wild-type animals.

In conclusion, this study shows that queuosine in tRNA, as opposed to free queuine base, is required for adequate tyrosine biosynthesis from phenylalanine and for maintaining appropriate levels of pterins and biogenic amines in the brain. The molecular basis of these changes are still uncertain but may be linked directly to tetrahydrobiopterin biogenesis or bioenergetic abnormalities.

Abbreviations

3-MT	3-methoxytyramine
5HIAA	5-Hydroxyindoleacetic acid
5HT	Serotonin or 5-hydroxytryptamine
5HTP	5-hydroxytryptophan
7-MG	7-methyl guanine
A	Adenine
AADC	Aromatic acid decarboxylase
aaRSs	Aminoacyl tRNA synthetases
ADHD	Attention-deficit/hyperactivity disorder
AdoMet	S-adenosylmethionine
ADP	Adenosine diphosphate
ARs	aldose reductases
Asn	Asparagine
Asp	Aspartic Acid
ATP	Adenosine triphosphate
BCIP	bromo-4-chloro-3-indolyl phosphate
BH ₄	Tetrahydrobiopterin
Bp	Base pair
BSA	Bovine Serum Albumin
C	Cytosine
Ca	Calcium
CaCl ₂	Calcium Chloride
CD	Chemically Defined
CD ₃ OD	Deuterated methanol
CDCl ₃	Deuterated chloroform
CE	Cerebellum
CNS	Central nervous system
CO ₂	Carbon dioxide
COMT	catechol- <i>O</i> -methyltransferase
CRs	Carbonyl reductases
CS	Citrate Synthase
CSF	cerebrospinal fluid
D ₂ O	Deuterium oxide
DA	Dopamine
D-arm	dihydrouridine arm
DcOH	Dimerization cofactor of Hepatocyte Nuclear Factor 1 α

DHFR	Dihydrofolate reductase
DHPR	6,7-Dihydropteridine Reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOMA	3,4-dihydroxymandelic acid
DOPAC	Dihydroxyphenylacetic acid
DOPEG	dihydroxyphenylglycol
dpm	Disintegrations per minute
DRD	Dopa-responsive dystonia
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E	Epinephrine or Adrenaline
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
eNOS	endothelial NOS
<i>et al.</i>	et alier (latin for 'and others
FAD	Flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FC	Frontal cortex
Fe	Ferrum (iron)
FMN	Flavin mononucleotide
G	Guanine
GABA	γ -aminobutyric acid
GST	Glutathione S-transferase
gti	genetrap inset
GTP	Guanine triphosphate
GTPCH	GTP cyclohydrolase I
G-tRNA	Guanine containing tRNA
H ₂ NTP	7,8-dihydroneopterin triphosphate
H ₂ O	Water
H ₃ PO ₄	Phosphoric acid
HC	Hippocampus
HCl	Hydrochloric acid
HClO ₄	Perchloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPA	Hyperphenylalaninemia
HPLC	High Performance Liquid Chromatography

HS	Horse Serum
HT	Hypothalamus
HVA	Homovanillic acid
hyg	HYG cassette
I	Inosine
IGF-1	Insulin-like Growth Factor-1
iNOS	inducible NOS
IPTG	Isopropylthio- β -D-galactose
ISH	<i>In situ</i> hybridization
K ₂ EDTA	EDTA dipotassium salt
K ₂ HPO ₄	Dipotassium phosphate
K _{cat}	First Order rate constant
KCl	Potassium Chloride
K _m	Michaelis-Menten constant
LB	Luria-Bertani
LCMS	Liquid Chromatography Mass-Spectrometry
LDH	lactate dehydrogenase
L-DOPA	3,4-dihydroxyphenylalanine
LNAA	Large Neutral Amino Acids
MAO	Monoamine oxidase
MBP	Maltose Binding Protein
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulfate
MHPG	3-methoxy-4-hydroxyphenylglycol
M-PER	Mammalian protein extract solution
mRNA	Messenger RNA
NA or NE	Noradrenaline or Norepinephrine
Na ₂ EDTA	EDTA disodium salt
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium Hydroxide
NBT	Nitroblue tetrazolium
ncRNA	Non-coding RNA
neo	B-geo
NGF	Nerve Growth Factor
NM	Normetanephine
NMR	Nuclear Magnetic Resonance
nNOS	neuronal NOS

NO	Nitric oxide
NOS	Nitric oxide synthase
oQ	Epoxyqueuosine
PAH	Phenylalanine hydroxylase
PAL	Phenylalanine Ammonia-Lyase
PBS	Phosphate Buffered Saline
PCD	Pterin-4a-carbinolamine dehydratase
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
pK _a	Acid dissociation constant
PKU	Phenylketonuria
PNMT	phenylethanolamine- <i>N</i> -methyltransferase
PPi	Pyrophosphate
PreQ ₀	7-cyano-7-deazaguanine
PreQ ₁	7-aminomethyl-7-deazaguanine
Psi	Pseudouridine
PTP	6-pyruvoyl-5,6,7,8-tetrahydropterin
PTPS	6-pyruvoyl-tetrahydropterin synthase
PVDF	Polyvinylidene fluoride
Q	Queuosine
Q-base	Queueine
Q-tRNA	Queuosine modified tRNA
<i>Qtrt1</i>	Queueine tRNA-ribosyltransferase 1
QTRTD1	tRNA-guanine transglycosylase domain containing 1
QueA	S-adenosylmethionine:tRNA ribosyltransferase isomerase
QueF	PreQ ₀ oxidoreductase
Qv1	QTRTD1 variant_1
RB	Rest of brain
RNA	Ribonucleic acid
RNase P	Ribonuclease P
rRNA	Ribosomal RNA
RTPCR	Real Time Polymerase Chain Reaction
<i>S. shibatae</i>	<i>Sulfolobus Shibatae</i>
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SR	Sepiapterin Reductase
ST	Striatum
T-arm	tRNA arm containing thymidine, pseudouridine and cytidine
TCA	Trichloroacetic acid

TGT	Transfer RNA Guanine Transglycosylase
TH	Tyrosine Hydroxylase
TLC	Thin Layer Chromatography
TNB	5-thionitrobenzene
TPH	Tryptophan Hydroxylase
Tris	Tris (hydroxymethyl) aminoethane
tRNA	Transfer Ribonucleic acid
tRNA ^{Ala}	Alanine tRNA
tRNA ^{Asp}	Aspartate tRNA
tRNA ^{His}	Histidine tRNA
tRNA ^{Tyr}	Tyrosine RNA
TSP	Trimethylsilyl propanoate
Tyr	Tyrosine
U	Uracil
UC	Ultraculture
UV	Ultraviolet
VHA	3-methoxy-4-hydroxymandelic acid
V _{max}	The velocity at maximum concentrations of substrate
wta	wild-type allele

Chapter 1

Introduction

1.1 RNA: a brief history

Arguably one of the most important discoveries in understanding life's molecular processes was the discovery of nucleic acids. In 1869 Johannes Friedrich Miescher isolated various phosphate-rich chemicals, which he called nuclein (now known as nucleic acids), from the nuclei of white blood cells.

This discovery was the first step towards elucidating the nature and function of nucleic acids. After this initial description in 1871 [1], other scientists started their investigations into nuclein. One such scientist, Albrecht Kossel, a winner of the Nobel Prize in Medicine, discovered that nuclein is composed of four bases and also contains sugar. In 1881, the botanist Eduard Zacharias was the first to establish a link between chromatin and the chemical substance nuclein, by showing that nuclein was an integral part of chromosomes [2]. The importance of this early work can be appreciated all the more when one considers that at the time, most scientists had a firm belief that complex proteins must be the carriers of genetic information. Proteins, it was reasoned, are comprised of 20 different amino acids, while DNA is only made up of 4 nucleotides – too few to store the enormous amount of genetic information.

Therefore, it was not until the 1940s and early 1950s that science once more became interested in nucleic acids. It was at this time that DNA was shown to be a carrier of genetic information through classic experiments carried out by Oswald T. Avery, Colin MacLeod and Maclyn McCarthy and Al Hershey with Marta Chase. A significant advancement came in 1950 with the publication of a role for RNA in protein synthesis by Torbjörn Caspersson, Jean Brachet and Jack Schultz [3]. In 1965, Hubert Chantrenne discovered that RNA acts as a messenger (messenger RNA) in the synthesis of protein [4]. Other important discoveries that followed were the demonstration that RNA forms a vital part of the ribosome (ribosomal RNA) and that other RNA molecules act as acceptors and carriers of amino acids in protein synthesis (transfer RNA).

RNA is a polymer that consists of a long chain of nucleotide units. Each unit consists of a nitrogenous base, a ribose sugar and a phosphate group which is attached to the 3' position of one ribose and the 5' position of the next. Although RNA consists of only four nuclear encoded bases (adenine, cytosine, guanine and uracil), numerous chemical modifications are known to occur on the base and sugar moieties of mature RNA [5], (Figure 1.1). Several types of RNA exist. Messenger RNA (mRNA) carries the genetic information transcribed from DNA to the ribosome in a linear form. The mRNA is read in sets of three-nucleotide sequences, called codons, each of which specifies a particular amino acid [6]. There are also so called non-coding RNAs ("ncRNA") which can be encoded by their own genes or derived from mRNA introns [7]. Many types of ncRNA, have roles in gene regulation, RNA processing, and the splicing and ligation of other RNA molecules [8-9]. Possibly, the most important ncRNA are transfer RNA (tRNA) and ribosomal RNA (rRNA), which together create the machinery necessary for translation. tRNA is involved in deciphering the codons in mRNA. Each type of amino acid has its own cognate tRNA, which binds to the amino acid and carries it to the growing end of a polypeptide chain. The decoding process ultimately relies on the presence of a three-nucleotide sequence within the tRNA called an anti-codon. This triplet specifically recognises and base-pairs to a complementary codon within the mRNA when presented in the context of the ribosome. The ribosome itself physically moves the mRNA molecule through its active site and in the process catalyses the assembly of amino acids into a polypeptide chain.

To fulfil all of these various cellular activities RNA needs much more functional complexity than provided for by its four canonical bases alone. The solution to this problem lies in the many different modifications found on RNA transcripts. In fact, *every* position of the purine (Figure 1.2) and the pyrimidine (Figure 1.3) ring can be post-transcriptionally modified as can the 2'hydroxyl group of the ribose moiety, through methylation or even ribosylation with a bulky adenosine-5'-phosphate group [10]. Around 110-119 naturally occurring modifications of RNA nucleosides [10] have been reported thus far, the most prevalent of which are base and ribose methylations (symbolised as mX and Xm respectively) and the isomerisation of uridine into pseudouridine (Psi). Pseudouridine is considered to be the most abundant modified nucleoside in RNA. The second most abundant modified nucleoside is 2'-O-methylribose

(Xm = Cm, Um, Gm, Am) [10]. Another nucleoside modification worth mentioning is inosine (I), which is formed when hypoxanthine (a product of adenine deamination) is attached to a ribose ring through a β -N₉-glycosidic bond. Inosine is commonly found in tRNA and plays a major part in providing 'wobble' or degeneracy to the tRNA decoding process, which will be discussed later. Despite knowing the structure and position of many modifications, the exact role of many of these naturally occurring modifications is still not well understood. However, many of these modifications occur in highly functional regions in the RNA molecule implying their importance in normal activity.

1.2 Transfer RNA Structure and Function

In 1965, the Nobel Prize winner in Physiology and Medicine, Robert Holley, reported the nucleotide sequence of yeast alanine tRNA (tRNA^{Ala}), the first nucleotide sequence of RNA ever to be determined. This was the initial step towards explaining how protein is synthesised from mRNA. Holley was the first to recognize that almost all tRNAs, which are generally between 74 to 92 nucleotides long, can be depicted as stem-loop secondary structure that resembles a cloverleaf. The three dimensional structure was later elucidated by Alexander Rich in collaboration with Sung Hou Kim and separately by Aaron Klung. They showed that the tertiary structure of tRNA conforms to an L-shaped arrangement [11] (Figure 1.4A).

The secondary structure of all tRNA molecules is stabilised by intramolecular hydrogen bonding and extensive base stacking interactions (Figure 1.4B). In considering the important functional elements, the tRNA molecule can be separated into four arms. The amino acid acceptor arm contains the free 3' and 5' ends of the chain positioned at the end of a 7 base pair stem. It is the 3'terminal OH to which an amino acid is attached. The 5'end phosphate may contain non-Watson Crick base pairs such as G-U (guanine-uracil). The second arm is called the dihydrouridine arm (D-arm). It contains a 3- to 4-base pair (bp) stem and the modified base dihydrouridine, from which the name is derived. It is believed that the main function of the D-arm is to facilitate recognition of the tRNA by its cognate aminoacyl-tRNA synthetase; the enzyme which attaches the amino acid to the tRNA molecule. In addition, the D-arm appears to have a role in stabilizing the tertiary structure of tRNA. The third arm is called the T ψ C or T-arm. It comprises a 5-bp stem, usually containing thymidine, pseudouridine (ψ) and cytidine residues in tandem. The function of the T-arm is to facilitate recognition by the ribosome during protein synthesis. Some tRNAs contain a so called variable arm, positioned between the anticodon stem-loop and the T-arm. Its length can vary from between 2 to 21 nucleotides and can contain a stem region of up to 7 bp.

The final structural element is called the anticodon arm. It is a 7bp loop that contains an anticodon of three nucleotides, always numbered 34, 35, 36, which correspond to the mRNA codon. Because of wobble base pairing, a non-Watson base interaction, some tRNA anticodons can pair with more than one codon on the messenger RNA. The four main wobble base pairings are G-U, inosine-uracil (I-U), inosine-adenine (I-A) and

inosine-cytosine (I-C). The ability to form a wobble base pair is fundamental to the proper translation of the genetic code. In all tRNAs there are 15 positions where the same base always occurs. There are also 8 positions, found principally in the loop regions that contain only a purine or only a pyrimidine base. Loop regions also contain correlated invariants which are pairs of non-stem nucleotides that are base paired in all tRNAs [12].

All mature tRNAs contain a terminal CCA sequence (positions 74-76) with a free 3'OH group where the amino acid is attached. In eukaryotes the CCA bases are not coded for by the tRNA gene. Instead, these nucleotides are added during processing of the pre-tRNA transcript. The aminoacylation by aminoacyl tRNA synthetase (aaRS) enzymes produces tRNA molecules with their CCA 3'ends covalently attached to a specific amino acid. This process of amino acid attachment to the 3'terminal ribose residue is driven by ATP hydrolysis in a two-step reaction. The first step is the reaction of the amino acid with ATP to form an aminoacyl-adenylate (see Figure 1.5A) and PPi. This reaction, in all but three aaRS enzymes, can occur in the absence of tRNA. The second step is the reaction between aminoacyl-adenylate and tRNA which results in the formation of aminoacyl-tRNA (see Figure 1.5B). This two-step reaction is then completed by the hydrolysis of PPi by inorganic pyrophosphatase.

The tertiary structure of tRNA was discovered by carrying out 9 years' worth of X-ray crystallographic efforts and showed that tRNAs folds into similar compact L-shapes [13-15] (Figure 1.4B). One of the helices containing the 5' and 3' ends is located on top of the helix that ends in the T ψ C loop to form one continuous arm; the other two helices combine to form another continuous arm. This structure is stabilised by base stacking and intercalation, and the formation of additional hydrogen bonds between different regions of the tRNA molecule. Modified nucleosides, magnesium and polyamines also participate in stabilising the tertiary structure of tRNA.

1.2.1 tRNA Post-translational Modification

There are several steps involved in tRNA maturation. tRNAs are synthesized from long precursor molecules called pre-tRNAs. First the 5'leader sequence is removed by Ribonuclease P (RNase P), a ribonucleoprotein complex [16]. RNase P was one of the

earliest catalytic tRNA molecules to be recognized [17]. Second, the 3' trailer sequence is removed by combination of endonucleases and exonucleases [12, 18].

Next, the CCA sequence is added, which is essential for amino acid attachment and interaction with the ribosome. The CCA sequence is synthesized *de novo* by a CCA-adding enzyme. The actual mechanism of how CCA-adding enzyme accomplishes the addition of CCA to the ends of tRNA in such a precise fashion is not well understood. An original model, published by Deutscher in 1972, depicted the CCA-adding enzyme as having three nucleotide-binding sites for progressive addition of the three nucleotides [19]. However, work done in the Weiner lab proposed a view that this protein has a single active site pocket and there is evidence that the tRNA substrate is fixed to the protein for the addition of the terminal C and A residues [16]. As well as this, another model exists that proposes the existence of two nucleotide binding sites, one catalytic CTP site and one ATP site that is both regulatory and catalytic [20], and a third, scrunch-shuttling model, exists which was proposed by Steitz and co-workers. This model is based on a tRNA-induced tetramer formation by the *S. shibatae* CCA-adding enzyme with only two tRNA molecules bound per tetramer. They proposed that two proteins of each tetramer have adjacent active sites that are responsible for C-addition and A addition, respectively [21].

In some tRNAs, introns are spliced by an endonuclease that excises the intron and a ligase that joins the exons. Studies carried out on the mutations in the secondary structure of tRNA showed that it is important for the process of intron excision [22]. There are a number of introns that are always found in the anticodon loop and since it is believed that pre-tRNAs are most likely to fold in the same way as mature tRNAs, this would bring the two introns-exon junctions close together. Since the misfolded tRNA precursors are not processed properly, the splicing of introns is believed to be a quality control mechanism in the generation of functional tRNAs.

And last but not least tRNA is extensively modified. There are 95 known post-translational modifications in the tRNA molecule (see Figure 1.6) and they have been found in all organisms studied so far. tRNA can be modified not only by methylation or hydroxymethylation, but also by deamination, transglycosylation, acetylation, reduction, thiolation, oxidation, ribosylation, formylation, isomerisation, selenation and multiple chemical group additions or transfers [10]. Due to this diversity in tRNA modifications

their functions are generally divided into two groups. The first group generally includes modifications that occur in the D and T ψ C loops and which are mainly involved in formation and stabilization of the tertiary L-shape of tRNA, playing an important role as internal molecular chaperones. The other group are modifications that occur in anticodon loop, which are important for the accurate and effective synthesis of proteins. These act by either altering codon recognition properties of tRNA, alteration of efficiency of decoding and reading frame maintenance or recognition by aminoacyl-tRNA synthetases [23]. Some anticodon loop modifications may have a dual function of precision in codon pairing and recognition by aminoacyl-tRNA synthetase enzymes.

In any particular tRNA, about 10% of the total number of nucleosides are modified, but it can involve up to 25% of the nucleosides [24]. Because there is such diversity in the types of modification of tRNA the enzymes involved are abundant. At least 45 different tRNA modification enzymes exist in bacterial cells and at least four times more genetic information is devoted to the synthesis of these enzymes than to the synthesis of RNA itself [25].

1.3 Introduction to Queuosine

Queuosine (Q) is a nucleoside with an unusual 7-deazaguanine core attached to a cyclopentenyl moiety via a methyl-amino linker. The nucleobase of queuosine is known as queuine or Q-base (7-((-4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanosine) (Figure 1.7A). The queuosine modification occurs on the wobble position of an anticodon loop of tRNAs coding for the amino acids histidine (His), tyrosine (Tyr), aspartic acid (Asp) and asparagine (Asn), whose anticodon loop sequence conforms to a GUN consensus sequences (positions 34-37; N is a symbol for any codon).

The queuosine modification is present in all eukaryotic and bacterial organisms with the exception of the yeast *Saccharomyces cerevisiae* and mycoplasma and is completely absent in Archaea, where it is replaced by archaeosine (position 15) [24]. Queuosine can be found as a free base, free nucleotide, free nucleoside and nucleoside incorporated into anticodon loop of tRNA. However, only bacterial organisms can synthesize queuine *de novo*. Eukaryotes obtain queuine from their diet, through tRNA turnover and from symbiotic bacteria resident in the gut.

Queuosine has been implicated in numerous physiological processes including differentiation, development, hypoxic stress management, tyrosine biosynthesis and protein synthesis. It was shown that in eukaryotic organisms tRNA is undermodified with queuosine during development *in utero* and is gradually lost through progressive stages of cancer development [12]. However, tRNA is completely modified with queuosine in mature non-dividing tissues [26].

1.3.1 Biosynthesis of queuosine

Based on experiments in *Salmonella typhimurium* it was established that the first step of queuosine biosynthesis is the conversion of guanine triphosphate (GTP) to 7-cyano-7-deazaguanine (preQ₀). This conversion is carried out by a GTP-cyclohydrolase-like enzyme, during which N7 and C8 atoms of the nucleotide base are removed and either an aminopropyl or cyanopropyl group, respectively, is incorporated in order for the ring to reform [27]. The preQ₀ molecule then undergoes reduction to 7-aminomethyl-7-deazaguanine (preQ₁) in a reaction catalyzed by the NADPH-dependent pre-Q₀ oxidoreductase (QueF); EC 1.7.1.13) [24, 28] (Figure 1.7B). The next two steps in the

bacterial biosynthesis of queuosine are carried out by two enzymes encoded in by a single operon [27]. The enzyme tRNA-guanine transglycosylase (TGT) incorporates PreQ₁ into tRNA in a base-exchange reaction with the elimination of guanine at position 34 of the anticodon loop. This enzyme has the ability to insert guanine and PreQ₀, but PreQ₁ is the preferred substrate. In the third and final step epoxyqueuosine is formed by S-adenosylmethionine:tRNA (AdoMet:tRNA) ribosyltransferase isomerase (QueA) [29-31], this is followed by reduction of the epoxide in epoxyqueuosine (oQ) in the presence of B₁₂ which is required to form queuosine [24, 32].

The mechanism of queuosine formation in eukaryotic species is significantly different. Unlike bacteria, eukaryotes cannot synthesize queuine *de novo* and therefore must obtain their queuine as a nutrient factor, from the gut flora or salvaged from the turnover of tRNA. The salvage pathway acts to maintain cytosolic levels of queuine when there is deficiency in the diet [33]. Once queuine is ingested, a presently undefined mechanism of intestinal, circulatory and cellular membrane transporters moves queuine from the extracellular space into the cytosol [27]. The free base of queuosine is then post-transcriptionally incorporated into the appropriate tRNAs, replacing guanine in the wobble position. This occurs in a single enzymatic step, where the glycosidic bond of the ribose sugar is broken. The formation of the queuosine modified base in the anticodon loop of tRNA is irreversible [34-35].

Recent studies carried out by our group show that queuosine formation in eukaryotic tRNA occurs via a mitochondrial-localized heteromeric transglycosylase [36]. This complex, in adults, is made up of TGT and a splice variant from the queuine tRNA ribosyltransferase domain containing 1 gene (QTRTD1). This result was confirmed by a follow-up study by Chen *et al.*, where the human QTRTD1 protein was co-expressed and co-purified with a human form of TGT [37]. Further experiments, including cross-linking, analysis by mass spectrometry and size exclusion chromatography demonstrated that the two proteins do indeed exist as a heterodimer [37].

From the studies carried out by our group it was found that of three splice variants of QTRTD1, Qv1 was in fact a predominant adult form [36]. By expressed-sequence tag analysis, the TGT and Qv1 mRNA was found to be always co-expressed in eukaryotic species. In mice, Northern blotting demonstrated a near identical expression pattern for both. It was also shown that a conservation of catalytic residues exists in the TGT subunit

of the eukaryotic enzyme with respect to its bacterial counterpart. Interestingly this study demonstrated that neither TGT nor Qv1 on their own had transglycosylase activity in *E.coli* cells with a *tgt* gene mutation. However, when they were combined *in vitro* the transglycosylase activity was detectable. It was suggested that TGT performs the core transglycosylation step during catalysis since when examined, Qv1 was found to have a clear sequence divergence from the active bacterial enzymes [36]. This was confirmed by the studies carried out by Chen *et al.*, by means of site-directed mutagenesis which demonstrated that the human form of TGT was indeed responsible for transglycosylase activity [37]. As for Qv1, it was suggested that it could act as a partner protein for TGT and it could also have a role in salvaging the queuosine 5'-phosphate nucleoside after normal tRNA turnover by cleavage of the 7-deazapurine-*N*-glycosidic bond [36].

Confocal and immunoblotting analysis suggested that TGT, probably through its association with Qv1, interacts with the outer mitochondrial membrane. Qv1 was shown to be stably associated with this organelle (Figure 1.8). By carrying out gel filtration studies it was demonstrated that no stable interaction between recombinant TGT and Qv1 exists and it was proposed that tRNA could be involved in bridging the two proteins during the catalytic reaction or that additional proteins may be needed. Since TGT requires being in contact with mitochondria-localised protein during its catalysis Q-modification of tRNA necessarily occurs in cytoplasm and not in the nucleus [36].

The importance of queuine to eukaryotes was highlighted in a paper by Walter Farkas, which showed that mice tenaciously retain queuine when it is no longer freely available from the diet. Eight-week germ-free (axenic) mice were placed on a queuine-free diet for 4 weeks duration. Although the queuosine levels in tRNA^{Asn} and tRNA^{His} decreased by 70%, the levels in tRNA^{Asp} and tRNA^{Tyr} remained at 100% [38-39]. In fact, it took one year for mice on this queuine-free diet to become depleted in queuosine in all four tRNA species. The results also prove that eukaryotic organisms cannot synthesize queuine *de novo* and rely exclusively on bacterial species to supply queuine.

1.3.2 Physiological Role of Queuosine

The biochemical function of the queuosine modification is not well understood, but a number of physiological roles for it have been implied. A paper published in 1980s by Farkas [38] showed that germ-free mice fed a chemically defined queuine-free diet had

no obvious physiological differences from the mice who had queuosine modified tRNAs. Another paper published in 1982 by Noguchi *et al.* [40] showed that *E.coli* that lack *tgt* gene appeared to be unaffected and their amino-acyl charging ability remained intact. However, *E.coli* harbouring a deletion of *tgt* gene are readily outcompeted by wild-type strains in competitive growth experiments and after a short period of time comprise just 1% of the control [41].

Mutational studies carried out on the human pathogen *Shigella flexneri* showed that TGT is a gene product of the *vacC* locus, a virulence-associated chromosomal locus associated with epithelial cell invasion. A mutant *vacC* containing a deletion of the *tgt* gene showed a significant reduction in the level of virulence and the introduction of a cloned *tgt* gene from *E.coli* was found to restore virulence [42]. In another paper by Durand *et al* [43] it was shown that *Shigella flexneri* mutants that lack queuosine modified tRNA again show a significant virulence reduction. In this paper, mutations of *tgt* gene product resulted in the reduction of *virF* levels to 10%, a key transcriptional activator involved in virulence gene expression and haemolytic activity to 50-60% when compared to wild-type. It was suggested that this is due to impaired translation since the levels of *virF* mRNA remained the same. Later, in another set of studies carried out by Hurt *et al.* [44] it was shown that TGT recognises *virF* mRNA and it has been suggested that it has a riboswitch binding motif.

The position of the queuosine modification in the anticodon loop of tRNA implies its function in the process of translation and codon recognition. In fact, queuosine modification is known to result in an increase of translational speed and correct frame selection due to an increase in the efficiency of interaction between the wobble base and specific mRNA [41, 45-47]. It has been shown that the rate of aminoacylation in L-M cells containing tRNA^{Asp} modified with queuine and unmodified tRNA varies [48]. The reaction rate for the aspartate incorporation into unmodified tRNA is half of that of queuosine modified tRNA. The conclusion drawn from this experiment was that the modified tRNA is a better substrate for the aminoacylation reaction than unmodified tRNA. Queuine modification also seemed to increase the affinity of synthetase enzyme for the substrate. The $V_{\max}/K_m$ ratio increased from 5 in unmodified tRNA to 12 in queuine modified tRNA [48].

In eukaryotic organisms, the levels of queuosine modification seem to be directly correlated to the stage in development [26]. Queuosine-tRNA appears to be undermodified during the early stage of development whereas tRNA in mature tissues is fully modified with queuosine. Partial queuine deficiency has also been shown in rapidly growing tissues such as embryonic tissues and regenerating rat liver [49-50]. A deficiency in queuine has been observed in placenta despite a large amount of queuine being present in amniotic fluid [51-52]. Emmerich *et al.* [53] had also showed that variability exists in the extent of queuine-deficient tRNA in human leukaemias and lymphomas. In this study it was demonstrated that the amount of queuine deficient tRNA in high-grade lymphomas is substantially greater than that observed in germinal centre cell lymphomas and chronic lymphocytic leukaemia and in non-neoplastic lymphoid tissues. Emmerich suggested that the extent of queuosine deficiency in tRNA can be correlated to the grade of malignancy [53].

In another study by Huang *et al* [54] an investigation was carried out into the significance of the deficiency of queuosine-tRNA in the grade of malignancies in human lung cancer. Total tRNA was extracted from twenty-three different lung cancer samples and analysed for queuosine content. It was shown that all of the lung cancer specimens contained tRNA without queuosine modification, however twelve specimens of healthy lung also contained queuine deficient tRNA. The level of queuosine free tRNA was found to be higher in lung cancer samples than that in normal lung tissue. It was also suggested that lower rate of survival exists in patients with higher level of queuosine deficient tRNAs and so can be used as a prediction of survival of patients with lung cancer [54].

In another study by Baranowski *et al* [55] tRNA was analysed for the level of queuosine modification in sixteen ovarian malignancies, six myoma samples that were taken as benign tumour references and four myometrium samples taken as healthy tissue references. In this work Baranowski was able to demonstrate that queuosine hypomodification of tRNA is significantly increased in well-differentiated tumours when compared to moderately differentiated tumours, which in turn had higher levels of queuosine deficient tRNAs to those found in poorly differentiated tumours.

The reason for hypomodification of tRNA with queuosine is to date unclear. It was suggested that the hypomodification of tRNA with queuosine might occur in tumours due

to the lack of TGT activity in cancer cells [56]. It could also be that the rate of queuine uptake by cancer cells is low and tRNA synthesis is high when compared to healthy cells or that queuine is competing with some unknown factors such as pteridines [51]. It was also shown by Langguth and Kersten [57] that in cancer cells low level of free queuine exists and hence is not as readily available for tRNA modification as in normal cells.

Langguth and his co-workers [58] carried out an investigation into the effect of queuine on proliferation and differentiation of HeLaS3 cells. It was shown that queuine promoted the proliferation of HeLa cells and seemed to inhibit the growth of cancer derived and transformed cells. It was previously shown that glycolysis seems to be increased in tumour cells [59] and that activation of some oncogenes results in metabolism shifting to glycolysis under aerobic conditions [60]. From this Langguth [58] deduced that queuine induces mammalian cell proliferation under conditions that favour respiration, while the shift of metabolism towards glycolysis results in inhibitory effect of queuine on cell proliferation. This implied a role in metabolic state of the cell. It was also demonstrated that the addition of queuine to cancer cells, which were deficient of queuine nucleoside in tRNA, resulted in the presence of queuosine-tRNA, which either increased or inhibited proliferation depending on which cell line it was. This suggested that the role of queuine in proliferation was not directly linked to presence or absence of queuosine-tRNAs. From this Langguth *et al.* [58] deduced that increase or inhibition of proliferation is dependent on the aerobic or glycolytic state of cells and on the presence of queuine as a free base. In 1994 Reisser, Langguth and Kersten [61] carried out studies to investigate the correctness of this deduction. The results of this work showed that it is free base queuine that is important in metabolic adaptation of HeLa cells to the hypoxic environment. It was shown that growing HeLa cells under hypoxic conditions resulted in increase of free queuine and absence of queuosine modified tRNAs. Reisser suggested that queuine accumulation in HeLa cells occurs against concentration gradient and that queuine uptake must happen by active transport. It was previously shown that the activity of TGT enzyme in prokaryotes and eukaryotes that is responsible for queuosine modification of tRNA is decreased *in vitro* under hypoxic conditions, suggesting the requirement of oxygen for normal TGT activity [62]. From this Reisser *et al.* deduced that the absence of formation of queuosine-tRNA and the abundance of free base queuine was due to decrease in the activity of TGT enzyme [61].

In this work by Reisser *et al.* [61] a comparison was done of cells grown in anaerobic and aerobic conditions. It was shown that mitochondria of cells grown under aerobic conditions had higher electron flow and lower activity of Lactate Dehydrogenase A₄ isoenzyme. Lactate Dehydrogenase (LDH) is an important enzyme that is involved in energy production of the cell by catalyzing a reversible inter-conversion of lactate to pyruvate. The amount of Lactate dehydrogenase present in tumour cells and hypoxic conditions in HeLa cells is high and this is due to high glycolytic conditions, which result in the formation of lactate. In contrast to the conditions in aerobically grown cells, hypoxic conditions caused an increase in LDH A₄ activity. Since no increase in queuosine-tRNA was observed it was concluded that the upregulation of LDH A₄ enzyme is caused by presence of free queuine. From the results obtained in this paper it was deduced that queuine is involved in the improvement of respiration and prevention of glycolysis in the presence of oxygen and protection of cells from hypoxic stress [61].

In a paper by Pathak and Vinayak [63] it is suggested that the presence of queuine decreases the rate of growth in high LDH conditions and conversely that queuine increases the rate of growth in low LDH conditions. Similar observations were made in cancerous DLA mice that have high levels of LDH present in their serum and which decrease to lower levels when queuine is added [63]. Our own laboratory's hypothesis, formed from this data and some of our own recent work, is that queuine may have a role in controlling the metabolic state of the cell and may possibly be part of a switching mechanism from anaerobic to aerobic metabolism.

A study done by Szabo *et al.* [62] also was suggested that queuine might have a role in oxidative metabolism in mice. In this study, murine fibroblast LM cells were grown in queuine deficient and sufficient conditions and it was shown that the activity of manganese-containing superoxide dismutase is 53% less than that found in LM cells grown under sufficient queuine conditions. It was also shown that the activity of enzymes involved in oxidative lipid metabolism was also decreased in queuine deficient LM cells.

1.3.3 The Link between Queuosine and Tyrosine

A paper published in 1997 by Marks and Farkas [64] provided evidence of a link between queuosine modification and the synthesis or downstream functions of the amino

acid tyrosine. However a significant amount of work needed to be carried out over a number of years before reaching this conclusion. In the first major study published in 1980, Farkas examined the effect that diet has on the queuosine family of tRNAs of germ-free mice [33]. In this paper Farkas showed that the absence of queuine and queuosine in the diet led to a decrease in the amount of queuosine-tRNA present. However this decrease was found only in tRNA^{Asn} and tRNA^{His}. The second study performed by Farkas's group was published in 1981 [39]. In this paper, by Reyniers et al, it was shown that mice could in fact be completely depleted of queuosine in all four tRNAs. This was done by placing germ-free mice on a CD diet for one year. It was also shown that this queuosine depletion of tRNAs had no adverse effect as these mice appeared normal. After depleting these germ-free mice of queuosine-tRNA the investigation was carried out into whether feeding or injecting queuine would reverse the disappearance of queuosine-tRNA. It was shown that both injection and feeding of queuine induced the synthesis of queuosine-tRNA that was proportional to the amount of queuine administered.

The next paper published in 1997 by Marks and Farkas [64] was able to provide us with an insight into the link between tyrosine and queuine. As in the experiments carried out in 1980 [33], Farkas placed germ-free mice on a queuine-free diet. This time mice were kept under stress-free conditions on this diet for the duration of two months. After this period of time, tyrosine was removed from their diet and it was substituted with phenylalanine since studies have shown that tyrosine is not a dietary requirement if an adequate amount of phenylalanine is present. Within the first three days most of the mice started showing poor health and within eighteen days all mice displayed a number of behavioural symptoms (Tables 1.1 & 1.2). The first symptom to appear was squinting, then their shiny white coats became scruffy, mice became lethargic, several mice died, one mouse had convulsions and died when it was gently handled. At this point in time some of the mice were placed on a tyrosine sufficient diet and the remaining continued to be maintained on a tyrosine-free diet. By day 17 only half of the mice left on a tyrosine-free diet remained alive and most of them exhibited convulsions in response to gentle handling and died, with the only remaining one naturally dying on day 18. All of the mice that had been placed back on tyrosine-sufficient diet had a complete recovery. This experiment was repeated again only this time chemically synthesized queuine was added

to the tyrosine-free diet of some of the germ-free mice. The administration of queuine prevented fatalities in the animals.

From these studies it appears that queuine is needed for viability under tyrosine deficient conditions, although to date the relationship between queuine and tyrosine biosynthesis/utilization is unclear. The most logical step would be to examine the effect of queuine on proteins that are involved in tyrosine biosynthesis. In eukaryotic organisms tyrosine is produced by hydroxylation of phenylalanine in a reaction catalyzed by the phenylalanine hydroxylase (PAH) enzyme [65]. PAH catalyzes the addition of a hydroxyl group to the end of the 6-carbon aromatic ring of phenylalanine, thus forming tyrosine. Tyrosine itself can affect the rate of phenylalanine hydroxylation, tyrosine degradation or the metabolic pathway of tyrosine in the cell [66].

Once produced, tyrosine can be hydroxylated by another enzyme called Tyrosine Hydroxylase (TH) to 3, 4-dihydroxyphenylalanine (L-Dopa), which is a rate-limiting step in the biosynthesis of catecholamines. PAH, TH and tryptophan hydroxylase (TPH), an enzyme that hydrolyses tryptophan to 5-hydroxytryptophan and is a rate limiting step in the biosynthesis of 5-hydroxytryptamine (serotonin), are all members of a family of amino acid hydroxylases that share a high degree of sequence similarity. The hydroxylation of all aromatic amino acids requires the co-factor tetrahydrobiopterin, commonly abbreviated as BH₄ (Figure 1.9).

Enzymatically, TPH, TH and PAH all incorporate one atom of oxygen into the substrate and the other atom is reduced to water. BH₄ supplies the electrons required (Figure 1.9). In higher eukaryotes PAH protein is mainly found in liver and provides an endogenous supply of tyrosine, making it a non-essential dietary component, and controls the levels of phenylalanine by catabolising it to carbon dioxide and water [67]. Both TH and TPH are involved in neurotransmitter biosynthesis and can thus be found in brain. All aromatic acid hydroxylases require the reduced form of BH₄, molecular oxygen and iron for their function. PAH, TH and TPH are regulated by slightly different mechanisms but in general they depend on their substrate, BH₄ and phosphorylation of serines [67]. Studies have shown that these enzymes are inhibited by catecholamines, biogenic amine neurotransmitters that are derived from an amino acid tyrosine, such as dopamine, noradrenaline and adrenaline [68]. TH and PAH were shown to be inhibited by direct co-ordination of catechols to the Fe³⁺ at the enzyme active sites. Phosphorylation of the

Ser40 residue in TH enzyme has also been shown to result in a dramatic decrease in the binding affinity of catecholamines and an increased affinity for BH₄ cofactor [67].

1.4 Phenylketonuria and Tetrahydrobiopterin

1.4.1 Brief Introduction to the Phenylalanine Hydroxylation System

The phenylalanine hydroxylation system consists of several components that each contributes to the regulation of tyrosine production. The first component is the PAH protein itself, which can exist as either a homodimer or homotetramer, and is found primarily in liver and kidney [69-71]

PAH consists of a three domain structure with a regulatory domain, containing an N-terminal autoregulatory sequence that is positioned over the active site, a catalytic domain that contains an active site iron and the substrate binding sites for BH₄ and phenylalanine, and a C-terminal oligomerization domain [72] (Figure 1.10).

Phenylalanine, obtained mainly by dietary means, is a positive effector or activator of PAH as it induces a conformational change in the enzyme. Oxygen is also necessary for the hydroxylation reaction. Studies in rodents showed that phosphorylation of Serine 19 residue in PAH seem to also contribute to its activation [73]. Then there is the cofactor BH₄ that gets recycled after being oxidized and can be synthesized *de novo* in eukaryotes. BH₄ is known as a negative effector as it competes with phenylalanine activation to form a dead-end complex (PAH.BH₄). Another BH₄ function is to prevent PAH misfolding and inactivation during its expression and it also has a role in protecting PAH from proteolytic cleavage [73]. The activity of the PAH protein is also regulated by pterin-4 α -carbinolamine dehydratase (an enzyme that catalyzes the dehydration reaction of oxidized pterin) and dihydropteridine reductase (an enzyme involved in recycling oxidised BH₄). PAH is not inhibited by its cofactor product, BH₄-4 α -carbinolamine, but it is inhibited by primapterin (7-dihydrobiopterin) a product formed non-enzymatically from BH₄-4 α -carbinolamine (Figure 1.9). Therefore, changes in PAH phosphorylation, cofactor supply/recycling, the eradication of toxic cofactor metabolite, in addition to PAH gene mutation, can contribute to phenylketonuria.

1.4.2 Introduction to Phenylketonuria

Phenylketonuria (PKU) also known as Følling's disease, is a common hereditary disease that is found in about 1 in 10,000 Caucasian (white) newborns, and in much less

frequency in infants of Japanese, Ashkenasi Jewish, Finnish, and African descent. The greatest number of affected individuals is in Turkey, where 1 in 2,600 newborns are affected. Ireland too has an uncharacteristically high number of cases, ~1 in 5,000 individuals.

PKU was first discovered in 1934 by Dr. Ivar Asbjørn Følling, a Norwegian physician and biochemist. He was approached by a woman whose young children developed mental retardation despite being born normal. She noticed that one of the child's urine developed a strong smell when he was about one year old. After carrying out a number of experiments on urine Følling was able to determine that the substance causing this strong smell was phenylpyruvic acid. This condition characterized by excess of phenylpyruvic acid in urine became known as PKU [74]. The most common cause for PKU is a deficiency in the PAH enzyme, due to a recessively inherited mutation in the PAH gene. Mammals have no ability to synthesize phenylalanine *de novo* it can only be obtained by dietary means. However, in adult human beings only 10% of phenylalanine consumed is required for anabolic processes. The other 90% of phenylalanine is hydroxylated to form tyrosine. When PAH activity is limiting the excess phenylalanine is eradicated from the body through urinary excretion of intact phenylalanine or its deamination product known as phenylpyruvic acid [75-76].

Presently, all new-borns are routinely screened for PKU within eight days after birth. If left untreated PKU leads to the development of mental retardation and neurological disturbances [77-78]. It has also been characterized by hyperactivity, hypoactivity, asthma, headache, hypopigmentation, seizures, microcephaly, growth failure and eczematous rash [75, 79-80]. To date the exact mechanism by which high phenylalanine concentrations can cause mental retardation had not been found. One of the suggestions has been that high phenylalanine levels negatively affect the myelation process in the brain which is needed for proper functioning of the nervous system [81-82]. Another mechanism that has been suggested is that the higher affinity for phenylalanine transport across the blood barrier causes competition for other amino acids such as tyrosine and tryptophan which are precursors for the biogenic amines dopamine and serotonin, respectively. Hence, the preference for phenylalanine could cause a disturbance in dopaminergic and serotonergic neurotransmission [82-83]. However, somewhat surprisingly, a number of PKU patients have been identified that did not develop mental

retardation and subsequent symptoms, even though their blood phenylalanine level was high and there was very poor dietary control. The explanation for this was found in near normal brain phenylalanine levels even though blood phenylalanine concentration was high. This suggests that the blood brain barrier is important for the pathogenesis of PKU. In fact a number of studies have shown there is great variability in blood phenylalanine versus brain phenylalanine levels in PKU patients [84].

1.4.2.1 Diagnosis and Treatment of Phenylketonuria

The diagnosis of PKU is based on the confirmation of a high blood serum concentration of phenylalanine (with reference values of 35 to 76 $\mu\text{mol/l}$) obtained on a normal diet following a positive newborn screening test [85]. It is crucial for PKU to be detected early in order to prevent mental retardation by dietary intervention. In the case of classical PKU the blood phenylalanine level reaches $>1200\mu\text{mol/l}$, which is indicative of a total depletion of PAH enzymatic activity. Mild/atypical PKU is characterized by phenylalanine blood levels of 600 to $1200\mu\text{mol/l}$ and hyperphenylalaninemia (HPA) is characterized by blood phenylalanine levels of 240 to $600\mu\text{mol/l}$ [86]. Normally, during development the phenylalanine of affected foetus is cleared by transplacental transport and so the affected baby is born with normal phenylalanine levels. However, once the baby is fed either mothers breast milk or formula feed for as little as 24 hours, it is possible to detect high enough phenylalanine level to provide a positive test result ($>120\mu\text{mol/l}$) [75].

At the moment the most successful method for newborn screening for PKU is tandem mass spectrometry, since this method allows screening for multiple disorders. This method is also cost effective and gives few false positives. Others include Guthrie bacterial assay, enzymatic testing, fluorometric and chromatographic analysis [75, 85]. The main objective for treatment of PKU is to lower blood phenylalanine concentration to a level that does not cause damage to the brain. The main PKU treatment at the moment is dietary protein restriction that is sufficient in all amino acids except phenylalanine. Views are varied on the optimal duration of this treatment by dietary means. Before 1970s, it was believed that this restriction in diet could be stopped at the end of childhood since maximal myelinisation occurs during childhood and so the toxic

effect of phenylalanine on the brain should not extend into adulthood. However, several studies suggested that elevated blood phenylalanine levels in adulthood could impair cognitive function, neuropsychological and psychiatric outcome [75]. On the other hand a paper by Channon et al [87] showed that discontinuation of the diet caused only subtle changes in cognitive functions when compared to patients who remained on the diet.

There can be a number of side effects caused by dietary treatment such as vitamin and micronutrient deficiency, osteoporosis, long-standing dietary deficiency of protein and calcium deficiency. This can be prevented by eating a completely normal diet with full protein and vitamin intake [75]. As an alternative to dietary treatment, phenylalanine ammonia-lyase (PAL) treatment can be used. PAL, a phenylalanine metabolizing enzyme is placed in the intestinal lumen where it can pre-digest phenylalanine obtained in the diet. It can also metabolize free phenylalanine that enters the intestine from endogenous bound pools into the non-toxic compound trans-cinnamic acid and ammonia, which can then in turn be easily converted into urea [84, 88]. Another method is to administer large neutral amino acids (LNAA) by oral ingestion. Since LNAA and phenylalanine share the same transport system, this creates competition with phenylalanine for neutral amino acid transporters on the intestinal enterocyte and at the blood-brain barrier and which in turn reduces phenylalanine absorption [89].

It was also discovered that for some PKU patients, daily oral administration of a synthetic form of BH₄, known as sapropterin dihydrochloride lowers blood Phe levels without altering dietary phenylalanine intake [90]. Hepatocytes contain active PAH enzyme as well as five other enzymes involved in the synthesis and recycling of BH₄. In some PKU patients, even though they had adequate control of their blood phenylalanine levels they still experienced PKU symptoms. These people were shown to have defects in either the recycling or synthesis of BH₄ [91].

The ultimate and definitive treatment for PKU would be a liver transplant, since for the greatest part phenylalanine metabolism takes place in the liver. Such a procedure was shown to be successful in a child with PKU, who obtained a liver transplant following the development of liver disease [76]. This resulted in complete correction of the metabolic phenotype [84].

1.4.2.2 PKU: Disorders and Deficiencies

Patients with PKU, treated from the early infancy exhibit normal intelligence, and have comparable motor speed, speech, language but their neuropsychological test scores appear to be slightly lower than those of people without PKU. The areas of higher brain function that seem to be affected include abstract reasoning, executive functioning and attention [75]. However, each can be improved by stricter PKU diet [92]. As well as this, abnormalities in white brain matter which are thought to result from a reduction in myelin were found by magnetic resonance imaging in adults who were continuously receiving treatment but had a relaxed diet [81, 93-95]. Again these abnormalities disappear when placed on a more severe PKU diet [75].

In patients who desisted from using the PKU diet after reaching adulthood there is a higher percentage of minor neurological symptoms including tremor, poor motor co-ordination and brisk tendon reflexes [93, 96]. As well as that, more serious neurological deterioration may occur, such as spastic paresis, late onset epilepsy, ataxia and tremor [75]. Psychiatric and psychological problems may also develop such as depression, social withdrawal, agoraphobia, anxiety, neurotic behaviour and low self-esteem [75, 79, 97]. The patients that were left untreated and subsequently developed mental retardation are known to exhibit psychiatric and behavioural problems [98]. These can sometimes be reversed by strict phenylalanine diet [99].

The PKU diet is based on a restricted protein intake and so this diet has to be supplemented with vitamin B12 and specific amino acids. The PKU patients who relax their diet upon reaching adulthood may experience Vitamin B12 deficiency since they tend to choose low protein products. Vitamin B12 deficiency can cause a number of clinical symptoms that can appear years after the start of poor Vitamin B12 intake. These include neuropathy, anaemia, dementia, depression, and psychoses [100]. Other disorders that can be caused as a result of PKU are osteoporosis, which is loss of bone mass caused by a deficiency in calcium, vitamin D, magnesium and other vitamins and minerals and osteopenia, which is thinning of the bone mass and is commonly seen in people above 50 years of age. This is one of the causes of morbidity and mortality of PKU patients in later life [75].

1.4.3 The physiological role of Tetrahydrobiopterin

The BH₄ can be found in all tissues of higher organisms and is essential for many different processes and functions. It acts as a cofactor for various enzymes and was proposed to have functions at a cellular level, such as a role in proliferative activity and as a trigger for apoptosis; it can also act as a generator or scavenger of reactive species [101]. The level of BH₄ can decrease dramatically during diabetes or coronary heart disease and the deficiency in biosynthesis or regeneration of BH₄ can lead to neurological abnormalities such as Dopa-responsive dystonia and it can cause severe depletion of monoamine neurotransmitters [101].

The best known and understood function of BH₄ is its role as a natural cofactor for PAH, TH and TPH. All three aromatic acid hydroxylases require dioxygen, iron and BH₄. After every catalytic turnover, the oxidative product of BH₄ is released and is subsequently regenerated in a BH₄ regeneration pathway. This oxidation product is formed in reactions catalysed by both PAH and TH. Studies carried out on PAH showed that it is activated when in reduced form. The reduction of PAH is believed to occur at a redox site, involving the enzyme's iron, during the oxidation of BH₄. One electron from BH₄ is used up to reduce Fe³⁺ to Fe²⁺ which is believed to be important in the reductive activation of PAH and the other electron is thought to go to oxygen.

BH₄ is also thought to act as a redox co-factor for all three forms of nitric oxide synthase (EC 1.14.16.2; NOS), inducible NOS (iNOS or NOS II), neuronal NOS (nNOS or NOSI), and endothelial NOS (eNOS or NOS III) [102]. These proteins show 50-60% sequence homology and structure similarity. NOS is a homodimer and each monomer contains an N-terminal oxygenase domain with binding sites for different cofactors such as NADPH, FAD, FMN and Ca²⁺/calmodulin. Two BH₄ molecules can be found at the dimer interface in the proximity of arginine substrate binding site and the heme moieties. In an NADPH- and O₂-dependent reaction catalyzed by NOS, L-arginine is hydroxylated to NO and L-citrulline. BH₄ also has a role in stabilising the dimer and promoting its formation, protecting against proteolysis and increasing arginine binding [101]. Studies carried out by Pastor and his co-workers [103] demonstrated that iNOS activity only requires a basal level of BH₄ synthesis for normal activity. However, if the levels of BH₄ are too low it results in NOS being uncoupled, leading to the generation of superoxide

and subsequently H_2O_2 , which can react with free NO to produce a neurotoxic peroxynitrate [101]. This mechanism first became apparent from studies done with eNOS and endothelial dysfunction in insulin-resistant diabetes.

On a cellular level BH_4 can both react with O_2 to produce free radicals and generate oxidative species including superoxide, H_2O_2 and peroxynitrite and, under different circumstances, it can also scavenge free radicals and prevent oxidative damage [101]. Two papers published in 1989 by Tanaka [104] and Kerler [105] reported that BH_4 seems to have proliferative activity in hemopoietic cells. A more recent study by Anastasiadis has shown that BH_4 together with sepiapterin enhanced the proliferation of SV-40 transformed human fibroblasts, rat pheochromocytoma (PC12) cells and rat C-6 glioma cells [106]. However, the later paper by Tanaka [107] showed that epidermal growth factor (EGF), nerve growth factor (NGF), and insulin-like growth factor-1 (IGF-1) increased the proliferation of PC-12 cells through an obligatory rise in intracellular level of BH_4 . Besides these functions that are attributed to BH_4 there are many others that are less defined, such as the requirement of BH_4 for neurotransmitter-releasing factor [108-110] and cell-mediated immunity and inflammation [111].

1.4.4 Biosynthesis of Tetrahydrobiopterin

Cells have the capacity to generate BH_4 by two distinct pathways: by the *de novo* biosynthetic pathway that uses GTP as a precursor and by the salvage pathway that depends on the availability of dihydropterins (Figure 1.9).

There are three main enzymes in the *de novo* biosynthetic pathway: GTP cyclohydrolase I (EC. 3.5.4.16; GTPCH), which carries out the committing step, 6-pyruvoyl-tetrahydropterin synthase (EC 4.6.1.10; PTPS), and sepiapterin reductase (EC 1.1.1.153; SR). This biosynthesis proceeds from GTP *via* the two intermediates 7,8-dihydroneopterin triphosphate (H_2NTP) and 6-pyruvoyl-5,6,7,8-tetrahydropterin (PTP) [101]. There is no sequence similarity between these three BH_4 enzymes, however in the polypeptide secondary structure the C-terminal domain of GTPCH shows a similar subunit fold with PTPS, whereas the overall SR structure is completely different.

GTPCH is the first enzyme in the biosynthetic pathway of BH_4 . Studies have shown that GTPCH is a rate limiting enzyme in rodents but not in humans [112-115]. It

catalyzes the conversion of GTP to dihydroneopterin triphosphate by opening the imidazole ring, releasing formate and carrying out Amadori rearrangement of the ribose [73] (Figure 1.9). The regulation of GTPCH activity occurs at both transcriptional and post-translational levels and GTP cyclohydrolase feedback regulatory protein (GFRP) [116].

GTPCH is always present in tissues such as liver and in brain regions important for monoamine neurotransmitter synthesis and is inducible in other cell types and organs. GTPCH activity is modulated by its substrate GTP, by BH_4 which acts as a feed-back inhibitor, and by phenylalanine which can act as a feed-forward stimulator and finally by calcium ions whose role still remains a mystery [101].

PTPS is the second enzyme in the pathway and it catalyzes the conversion of neopterin triphosphate to 6-pyruvoyl tetrahydropterin in an Mg^{2+} dependant, two-step reaction, involving a stereospecific reduction by an internal redox transfer, oxidation of both side-chain hydroxyl groups, and unusual triphosphate elimination [73] (Figure 1.9). Studies have shown that mRNA of PTPS in higher mammals is constitutively present but the protein and its activity is not. Its expression seems to be tightly regulated by yet unknown mechanism at a transcriptional level. It is a rate limiting step in BH_4 biosynthesis, at least in humans, its activity is much lower in humans than in rodents [101].

In the final step of BH_4 biosynthesis SR catalyzes the conversion of 6-pyruvoyl tetrahydropterin to BH_4 in an NADPH-dependent, two step reduction. The first NADPH-dependent reduction at a side-chain keto group leads to the formation of 1'-OH-2'-oxopropyl tetrahydropterin (Figure 1.9). The internal rearrangement of another keto group via side chain isomerisation results in a formation of the 6-lactoyl tetrahydropterin which is then reduced in a second NADPH-dependent step to BH_4 [73]. SR is found in all mammalian tissues and its expression is not limited in any way. It is expressed in all regions of the brain unlike GTPCH and PTPS which seem to be localized to monoamine neurons [101]. SR also has ability to participate in the pterin salvage pathway by converting non-enzymatically generated sepiapterin to 7,8-dihydrobiopterin (BH_2), which is then converted into BH_4 in a second NADPH-dependent reduction step by an enzyme called dihydrofolate reductase (EC 1.5.1.3; DHFR)[101] (Figure 1.9).

Other chemical routes exist for the final two steps of BH₄ biosynthesis involving alternative carbonyl reductases (CRs) and/or aldose reductases (ARs). AR and CR have an ability to convert 6-pyruvoyl tetrahydropterin to 6-lactoyl-tetrahydropterin, or 1'-OH-2'-oxopropyl-tetrahydropterin to BH₄ in NADPH-dependent reactions. CR also converts 6-pyruvoyl-tetrahydropterin to 1'-OH-2'-oxopropyl-tetrahydropterin and SR can isomerize 6-lactoyl-tetrahydropterin and 1'-OH-2'-oxopropyl-tetrahydropterin [73] (Figure 1.11).

1.4.5 Regeneration of the Tetrahydrobiopterin Cofactor

Regeneration of BH₄ cofactor is essential in liver since *de novo* biosynthesis of BH₄ is not sufficient for controlling of levels of phenylalanine. The first step of the BH₄ recycling pathway is the oxidation of BH₄ to BH₄-4 α -carbinolamine a reaction involving a molecular oxygen transfer to an amino acid by aromatic acid hydroxylases. BH₄-4 α -carbinolamine then acts as a substrate for dehydration catalyzed by an enzyme known as pterin-4 α -dehydratase (EC 4.2.1.96; PCD/DcOH) and is subsequently reduced to BH₄ by dihydropteridine reductase (EC 1.6.99.7; DHPR) (Figure 1.9).

PCD is found mainly in liver, but was also purified from kidney, brain, skin and hair follicle. It was first discovered as a contaminant during a purification of rat PAH enzyme and it has an ability to stimulate PAH activity. It catalyzes a dehydration of 4 α -hydroxy-tetrahydrobiopterin to quinonoid dihydrobiopterin. While quinonoid dihydrobiopterin acts as a strong inhibitor of PCD, BH₂—a non-enzymatic product of quinonoid dihydrobiopterin does not. Dehydration of BH₄-4 α -carbinolamine can occur non-enzymatically if there is a deficiency in PCD, but the rate of this reaction is insufficient to maintain BH₄ in a reduced state. As a result, a non-enzymatic reaction occurs where BH₄-4 α -carbinolamine is rearranged to form 7-dihydrobiopterin that is then in turn excreted in urine.

The mammalian PCD can be found in two forms: as a cytoplasmic homotetramer which is characterised by dehydratase activity and four independent active sites for recycling BH₄-4 α -carbinolamine, and as a nuclear heterodimer (α 2 β 2) with the transcription factor HNF α . Since PCD was discovered independently as an enzyme and transcriptional activator, it has also been named dimerization co-factor for HNF- α .

(DcOH). DcOH forms a heterotetrameric complex with the N-terminal dimerization domains of the HNF- α . Surprisingly, the HNF1 α :PCD transcription complex can bind to the PAH promoter and enhance transcription of the gene [117]. The importance of this activity was demonstrated by deletion of the HNF1 α gene resulting in animals exhibiting severe hyperphenylalaninemia [119].

In the final step of the BH₄ regeneration pathway quinonoid dihydrobiopterin is converted to BH₄ by a dimeric enzyme DHPR. In the course of this NADH-dependent reaction one molecule of water is released and the product quinonoid dihydrobiopterin is reduced back to BH₄. This reduction reaction involves a direct transfer of hydride from the reduced nicotinamide ring to the quinonoid dihydrobiopterin by DHPR [101]. DHPR is widely found in tissues of mammals and it is not only important for the regeneration of BH₄. Studies suggest that in brain DHPR works together with DHFR in the presence of NADH to maintain tetrahydrofolate levels [73].

1.4.6 The role of 7,8-Dihydrobiopterin

In the regeneration pathway of BH₄, quinonoid dihydrobiopterin is formed as a result of hydroxylation and is required by DHPR enzyme to complete the final step of BH₄ regeneration. However, quinonoid dihydrobiopterin is an extremely unstable compound and is readily tautomerized to BH₂, which is not a substrate for DHPR. Unlike DHPR, the enzyme dihydrofolate reductase (DHFR) has the ability to regenerate BH₄ from BH₂. This was originally discovered from the work of Hasegawa, H. et al [118] where they administered sepiapterin, BH₂, and BH₄ to mice and demonstrated that each treatment gave a comparable raise in BH₄ levels within the organs tested. They concluded from this study that BH₄ has a 'salvage pathway' that uses BH₂ as a key intermediate.

BH₂ has also been implicated in eNOS uncoupling but the role of DHFR in this process was not clear [119]. In the vasculature, NO (nitric oxide) derived by eNOS has an important role in physiological regulation of vessel tone and inflammation [120]. At the onset of diabetes and other chronic vasoinflammatory conditions, oxidation of BH₄ can result in deficiency of this cofactor and uncoupling of eNOS which results in formation of superoxide from nitric oxide [121]. However, both BH₂ and BH₄ were found to have equal affinity for binding eNOS and BH₂ has an ability to rapidly and efficiently replace

BH₄ in preformed eNOS-BH₄ complexes [121]. In a paper by Crabtree et al., [121] an investigation was carried out into whether uncoupling of eNOS is simply a consequence of BH₄ insufficiency or whether it occurs as a consequence of diminished ratio of BH₄ versus BH₂. It was shown that during diabetes, NO insufficiency is characterised by lower levels of BH₄ versus BH₂ ratio rather than BH₄ depletion *per se*.

In 2009 a study was where the role of DHFR in regulating BH₄ versus BH₂ levels was investigated in endothelial cells and in cell lines expressing eNOS combined with tet-regulated GTPCH expression [119]. This was done in order to compare the effects of low or high levels of *de novo* BH₄ biosynthesis. In this study it was suggested that DHFR may have a key role in eNOS uncoupling by maintaining proper BH₄ to BH₂ ratio, particularly when there is a deficiency in bipterin.

Some clinical studies were also carried out where investigation into plasma levels of pteridines was done in patients with cardiovascular disorders [122-124]. In one such study, which looked at chronic renal failure, it was shown that BH₄/BH₂ ratio was reduced depending on the severity of the renal failure [122]. In another study done by Shinozaki et al [123] it was shown that the plasma BH₄/BH₂ ratio was lower in patients with insulin resistance.

Another work, published in 2009 by Takeda, M. [125] showed the importance of measurement of BH₄/BH₂ ratio in patients with endothelial dysfunction. They have shown that plasma BH₂ levels increased, while BH₄ levels did not change relative to the increase in total number of coronary risk factors. So BH₄/BH₂ ratio decreased with the increase in total number of coronary risk factors. Therefore BH₄/BH₂ ratio can be used as a biomarker of endothelial dysfunction.

The superoxide formed from the action of uncoupling of eNOS plays a major role in the pathogenesis of cardiovascular diseases [126]. Several antioxidants exist that have the ability to reverse endothelial dysfunction in patient with coronary artery disease [127-128]. It has been shown that folates possess such an antioxidant potential [129] and it was suggested that folates may stimulate regeneration of BH₄ from the inactive oxidized quinoid dihydrobiopterin [130]. Clinical studies carried in children with BH₄ deficiency showed a considerable improvement [131].

It was also suggested that BH₂ together with sepiapterin potentiate superoxide formation during the uncoupled reaction of NOS in patients with BH₄ deficiency

disorders. This resulted in a significantly reduced level of NO in the brain of these patients [132-134]. The patients with sepiapterin reductase deficiency, in which both BH₂ and sepiapterin accumulate in the brain have been shown to have low levels of nitrate and nitrite (end products of NO) in cerebrospinal fluid and the administration of low-dose BH₄ treatment did not restore brain's normal NO production. So the uncoupling of NOS reaction is promoted by both oxidation of BH₄ to BH₂ and sepiapterin formation, which in turn stimulates production of superoxide and peroxynitrite [135]. BH₂ is formed by reduction of sepiapterin by sepiapterin reductase in endothelial vessels. However if the ability of DHFR to reduce BH₂ to BH₄ is too low this may result in formation of toxic metabolites, such as superoxide [135].

1.4.7 The Tetrahydrobiopterin Disorders and Deficiencies

The disorders of BH₄ synthesis and regeneration were first discovered in patients with hyperphenylalaninemia (HPA) who did not respond to a treatment with a low-phenylalanine diet and developed progressive neurologic symptoms before dying at an early age. During the course of their disease these patients maintained a high tolerance for phenylalanine and normal PAH activity. These atypical symptoms led to a belief that there was another form of HPA, probably due to a defect in the metabolism of BH₄. Since BH₄ is an essential cofactor for TH and TPH, in addition to PAH, a defect in BH₄ synthesis or regeneration leads to a deficiency of the neurotransmitters L-dopa, noradrenaline, adrenaline, and serotonin [73].

Today BH₄ deficiency is divided into two groups. One is the classical form of BH₄ deficiency; a monoamine neurotransmitter deficiency concomitant with HPA, the other is dopa-responsive dystonia (DRD) or SR deficiency, which is a monoamine neurotransmitter deficiency without HPA. The classical form can be characterised by deficiency in neurotransmitters dopamine and serotonin, and progressive neurological symptoms. It can be caused by mutations in genes of the proteins involved in BH₄ biosynthesis or regeneration and they are all inherited autosomal-recessively. DRD and SR deficiencies occur because of deficiency in BH₄ without HPA. DRD is thought to be caused by autosomal-dominant mutations in the *GCH* gene and DNA mutations can also be found in 40% of the patients. SR deficiency is inherited in an autosomal recessive

fashion and displays all biochemical characteristics that are found exclusively in neurotransmitter metabolites and pterins in the cerebrospinal fluid (CSF) [101].

Low levels of BH₄ are found in many other neurological diseases that do not have HPA such as Parkinson's disease, depression, autism and Alzheimer's disease. Other disorders with deficiency in BH₄ in human epidermis are skin diseases such as vitiligo and Hermansky-Pudlack syndrome, both of which involve lower PCD/DcOH activities, accumulation of 6- and 7-biopterin and H₂O₂ in skin, tyrosinase inhibition, and abnormal melanin biosynthesis [101].

There is another disorder that displays mild phenylketonuria. Patients that are affected by this disorder have autosomal mutations in the gene that encodes PAH but their BH₄ synthesis and regeneration is normal. This disorder responds to a treatment with oral BH₄, as an alternative to low-phenylalanine diet [101].

1.4.8 The Detection of Tetrahydrobiopterin Deficiencies

BH₄ is an essential cofactor for all three hydroxylases involved in the biosynthetic pathways of biogenic amine neurotransmitters. Therefore the deficiency of BH₄ in patients presents with symptoms of progressive mental and physical retardation, central hypotonia and peripheral spasticity, seizures and microcephaly. The BH₄ deficiency disorders can be divided into two forms: a severe form and mild (peripheral, partial) form. Today in order to be able to distinguish between the two forms the levels of neopterin, biopterin, and the neurotransmitter metabolites, 5HIAA (5-Hydroxyindoleacetic acid), a metabolite of serotonin, and HVA (homovanillic acid) in cerebral spinal fluid are determined. 5HIAA and HVA can easily be quantified by using high pressure liquid chromatography (HPLC). The levels of 5-HIAA and HVA dramatically decrease in patients with severe forms of BH₄ deficiency and are unaffected in mild forms of GTPCH, PCD and PTPS deficiencies. The ratio of HVA/5HIAA is a good indication of whether the deficiency in BH₄ is of the mild or severe form. This ratio is much lower in patients with GTPCH deficiency than in DHPR and PTPS deficiencies. In patients with GTPCH deficiency biopterin levels are always higher than those of neopterin. In the mild form of DHPR the 5HIAA levels decrease while HVA levels remain the same [112].

Diagnosis of these deficiencies requires measurement of pterin metabolites in urine, dihydropteridine reductase in blood spots, neurotransmitters and pterins in CSF. The outcome of these diseases is not lethal if the therapy is started quickly to reduce phenylalanine levels and replacement therapy for the lowered neurotransmitters initiated [136].

1.4.9 Biosynthesis of Biogenic Amines

Dopamine (DA), norepinephrine (noradrenaline; NE or NA), epinephrine (adrenaline; E), histamine, γ -aminobutyric acid (GABA) and serotonin (5-hydroxytryptamine; 5HT) are hormones and/or neurotransmitters derived from amino acids. The biosynthesis of all of these biogenic amines involves decarboxylation of the corresponding amino acid.

Only 1-2% of the serotonin in the whole body is found in the brain. It is present mainly in cells that are not neurons, such as platelets and mast cells, but similar to histamine it cannot cross the blood-brain barrier so brain cells must be able to synthesize it *in situ*. The important element in serotonin production is the presence of the amino acid tryptophan. In the first step of the serotonin biosynthesis tryptophan is hydroxylated at the 5 position to form 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (Figure 1.12). This enzyme absolutely requires molecular oxygen, pteridine cofactor and sulfohydryl-stabilizing substance such as mercaptoethanol to remain active *in vitro*. In the second step of serotonin production 5-HTP is almost immediately decarboxylated to serotonin by an enzyme that is almost identical to an aromatic acid decarboxylase (AADC; EC 4.1.128) [6]. The hydroxylation of tryptophan by 5-HTP is a limiting step in this pathway.

Dopamine, norepinephrine and epinephrine are considered to be catecholamines[11]. The catecholamine refers to all organic compounds that contain a catechol nucleus (benzene ring with two adjacent hydroxyl substituents) and an amine group. The biosynthesis of catecholamines is carried out in the brain, chromaffin cells, sympathetic nerves and sympathetic ganglia from their amino acid precursor tyrosine. In the first step of catecholamine synthesis, tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA) by TH in a reaction that requires oxygen as a co-substrate and BH_4 as a cofactor (Figure 1.12). This is a rate limiting step in the biosynthesis of the NE in the peripheral

nervous system and of NE and DA in the brain. Another enzyme that is thought to be linked to the TH step is DHPR. It catalyzes the reduction of quinonoid dihydropterin, which has been oxidized during the hydroxylation of tyrosine to L-DOPA. Since TH is an enzyme that requires reduced pteridines for its function and alteration in the activity of DHPR affects TH activity [6].

In the second step L-DOPA is decarboxylated to dopamine by an enzyme called DOPA-decarboxylase. Studies have shown that this enzyme acts not only on L-DOPA but also on tyrosine, histidine, tryptophan, phenylalanine as well as 5-hydroxytryptophan and so this enzyme became known as “L-aromatic acid decarboxylase”. This protein is the most active enzyme in the biosynthetic NE pathway and requires pyridoxal phosphate (vitamin B₆) as a cofactor [6]. In the next step, a second hydroxylation of dopamine leads to the formation of norepinephrine. The enzyme responsible for this step is called dopamine- β -hydroxylase. This enzyme requires molecular oxygen and ascorbic acid as a cofactor and can be stimulated by dicarboxylic acids such as fumaric acid although it is not an absolute requirement. In the final step, *N*-methylation of norepinephrine by an enzyme called phenylethanolamine-*N*-methyltransferase (PNMT) produces epinephrine[6]. The production of any one specific catecholamine depends on the presence or absence of enzymes that are involved in this pathway [137].

1.4.10 Properties of Biogenic Amines

Serotonin is located in the neurons of the raphe region within the brainstem, which have widespread projections to the forebrain and exhibit spontaneous monotonic activity in a regulated pacemaker pattern (Figure 1.13). Serotonin is believed to have a role in sensory and motor function during different behavioural states. Studies have shown that serotonergic activity is highest during waking arousal, lower during quiet walking and the lowest or absent during sleep [6].

Dopamine can be found in several brain regions but the major dopamine-containing region is the corpus striatum, which receives signals from substantia nigra (Figure 1.13). In fact dopamine synthesis in the striatum completely depends on the rate of impulse flow from the nigrostriatal pathway. When impulse flow is high the activity of the tyrosine hydroxylase enzyme is increased and more tyrosine is hydroxylated, which in turn

increases the affinity for BH₄. Dopamine is thought to be released from the nerve terminal in a calcium dependent manner due to invasion of an action potential and by presynaptic release-modulating autoreceptors [6]. The released dopamine can be retrieved by the nerve terminal and converted to dihydroxyphenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO) or it can be converted to homovanillic acid (HVA), at an extraneuronal site, by catechol-*O*-methyltransferase (COMT) and MAO. In rodents DOPAC is a major metabolite and both DOPAC and HVA are found in free or sulphate-conjugated form. In humans, however, it is HVA that is a major metabolite, and the amounts of the sulphate-conjugated form are very small. Another metabolite that is present in central nervous system (CNS) is 3-methoxytyramine (3-MT). Dopamine is believed to have a major function in controlling body movements [6].

Studies have shown that NE is synthesized mainly by the sympathetic ganglia since it is a major peripheral transmitter in this part of the visceral motor system (Figure 1.13). It is used by the locus coeruleus, where it is thought to influence sleep and wakefulness, attention and feeding behaviour [137]. Studies have shown that NE is not uniformly distributed in the nervous system and suggest that NE is serving some specialized function. In brain NE can be converted to an aldehyde intermediate called 3,4-dihydroxyphenylglyrenaldehyde by MAO, which then can be reduced by aldehyde reductase to 3,4-dihydroxyphenylglycol (DOPEG) or be converted to 3,4-dihydroxymandelic acid (DOMA) by aldehyde dehydrogenase that is then by action of COMT is converted to 3-methoxy-4-hydroxymandelic acid (VHA) and DOPEG is converted to 3-methoxy-4-hydroxyphenylglycol (MHPG) by COMT. Another route for the metabolism of NE is its conversion to normetanephrine (NM) by COMT, NM being converted by MAO to 3-methoxy-4-dehydroxyphenylglycoaldehyde, which is then reduced by aldehyde reductase to VMA [6].

Epinephrine is present in the brain at much lower levels than the rest of catecholamines, but it seems to have discrete anatomic distribution and is believed to have functions different to those of other catecholamines, but which to date have not been elucidated fully [6].

1.5 Aims of the Project

The work carried out by Marks and Farkas [38, 64] demonstrated that queuine has the ability to prevent lethality and other symptoms in germ-free mice that had been maintained on a tyrosine free diet. The aim of this project is to determine if and how free queuine or queuosine modified tRNA is linked to tyrosine production and whether there is similarities to the human metabolic disease phenylketonuria. To this end, the project focused on changes to the expression and activity of the PAH enzyme and changes in the metabolite patterns of the PAH cofactor BH₄.

The aims of this project were to:

1. determine if queuine is required for tyrosine production in human cell lines.
2. examine whether free queuine or queuosine modified tRNA is linked to tyrosine production using gene trap animals deficient in queuosine-tRNA.
3. investigate if queuosine-tRNA deficiency has an impact upon mouse behaviour and metabolism

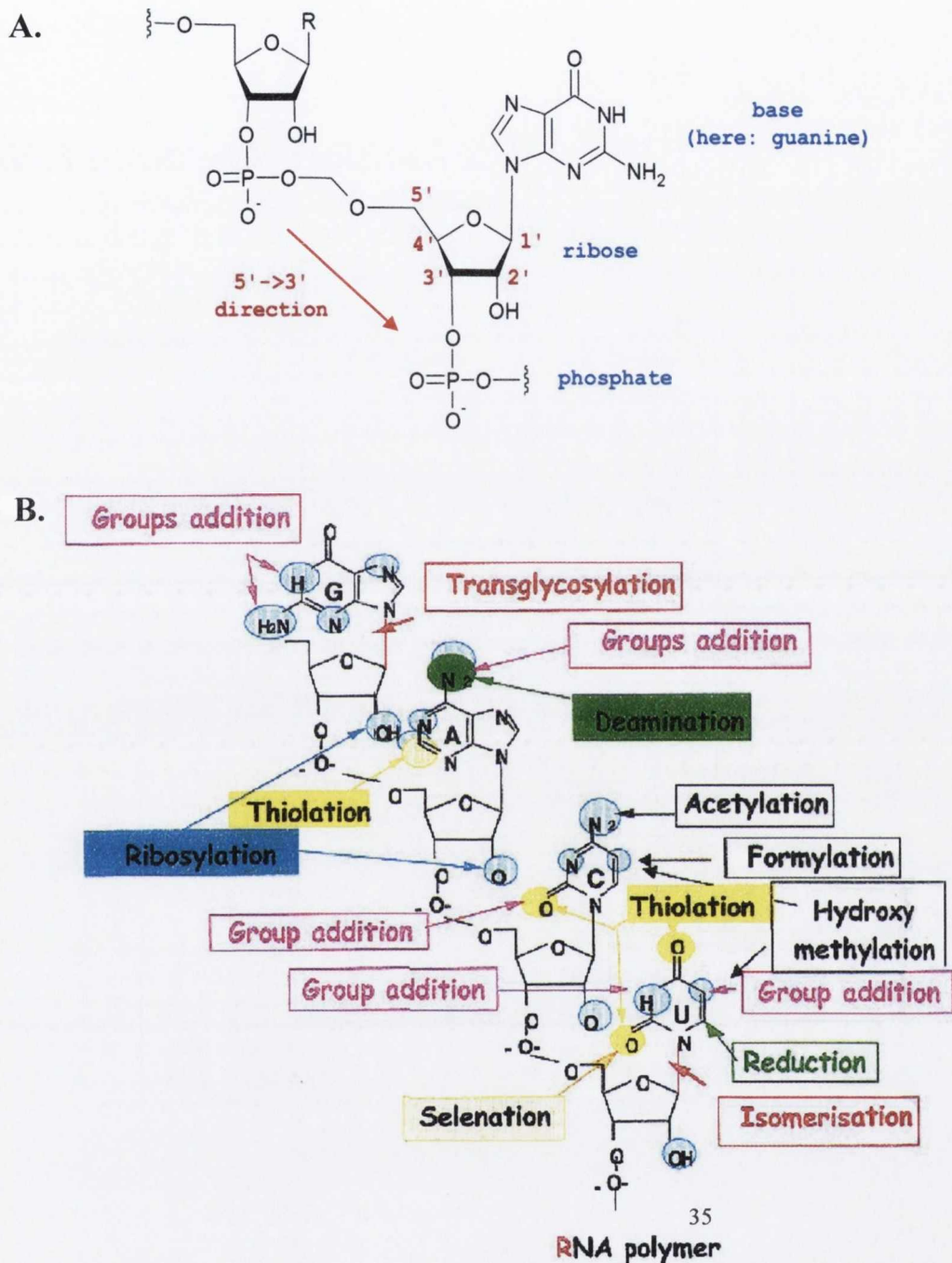


Figure 1.1 Structure and modifications of RNA molecule **A.** Chemical structure of RNA molecule. RNA is built up of a nitrogenous base, a ribose sugar, and a phosphate. The bases used are adenine (A), cytosine (C), guanine (G) and uracil (U). **B.** Overview of chemical modifications that can be found in tRNA molecules.

Purine derivatives in RNAs

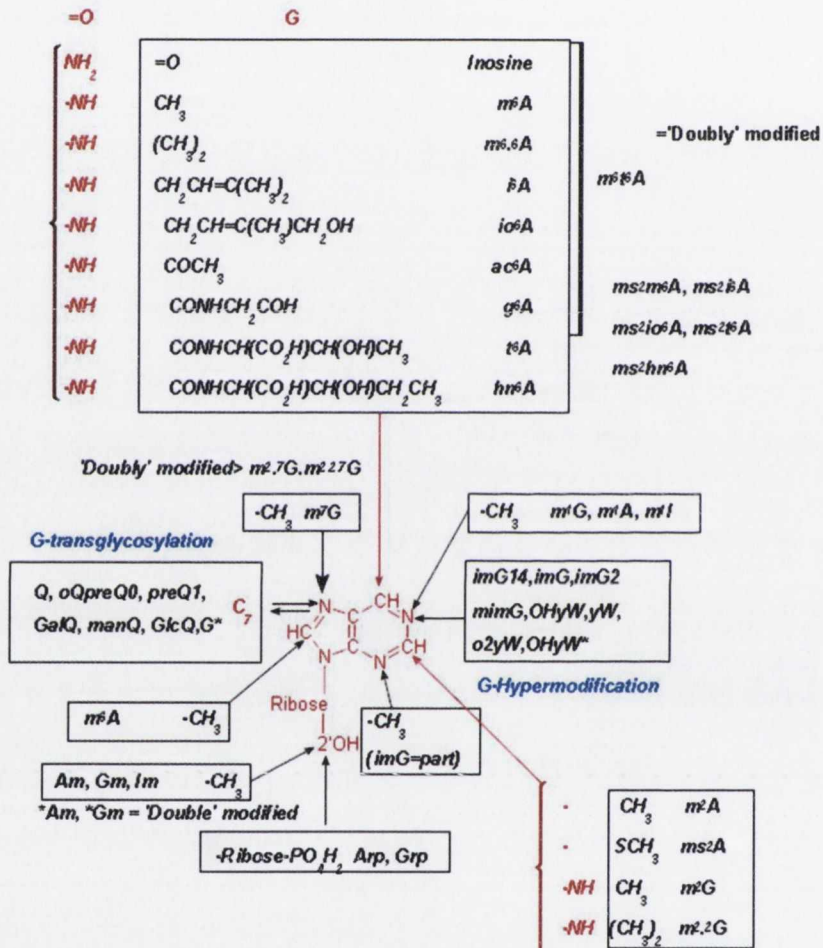
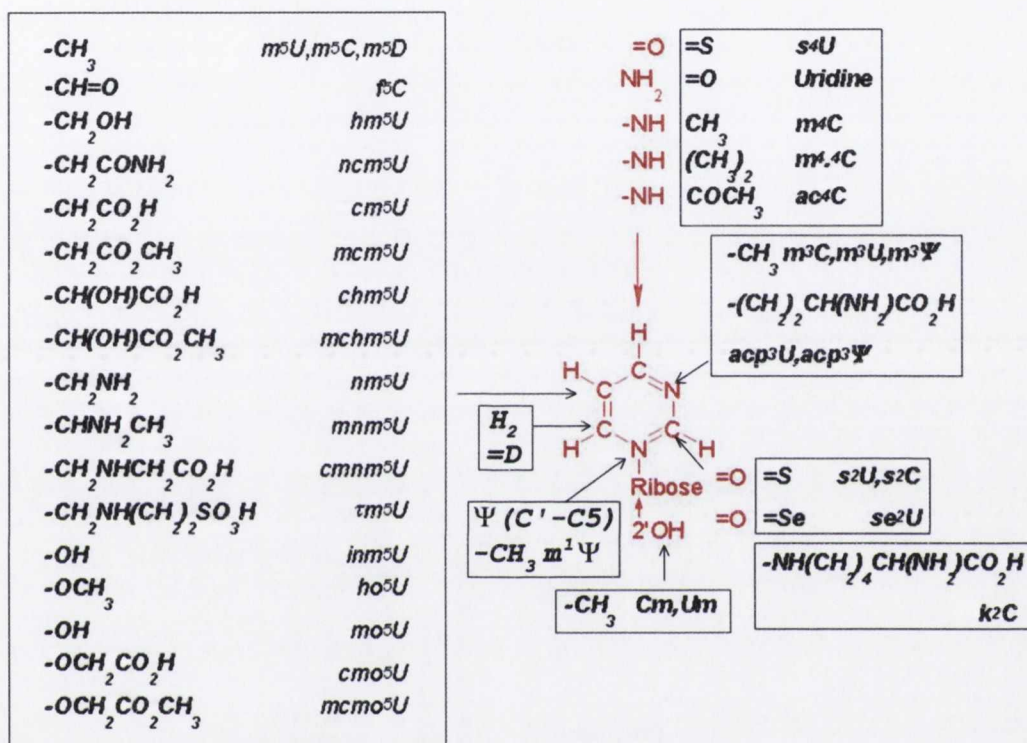


Figure 1.2 Modified bases and ribose in RNAs. In the boxes are the various types of chemical groups that can be enzymatically attached to selected atoms of a purine ring during maturation of RNA precursors in Bacteria, Eukarya or Archaea (Adapted from Grosjean et al [10]).

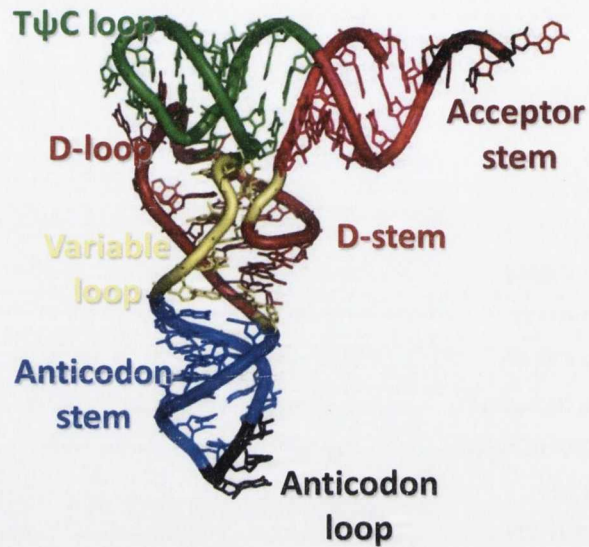
Pyrimidine derivatives in RNAs



* Cm, *Um = 'Doubly' modified

Figure 1.3 Modified bases and ribose in RNAs. In the boxes are the various types of chemical groups that can be enzymatically attached to selected atoms of a pyrimidine ring during maturation of RNA precursor in Bacteria, Eukarya or Archaea (Adapted from Grosjean et al [10]).

A.



B.

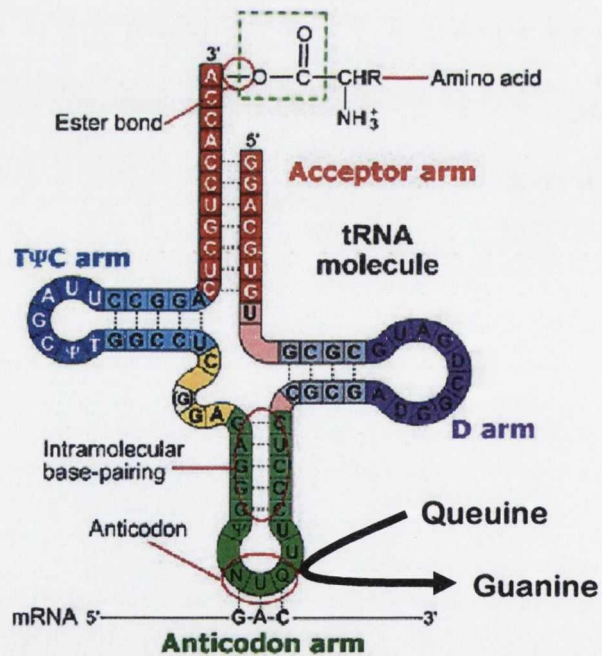
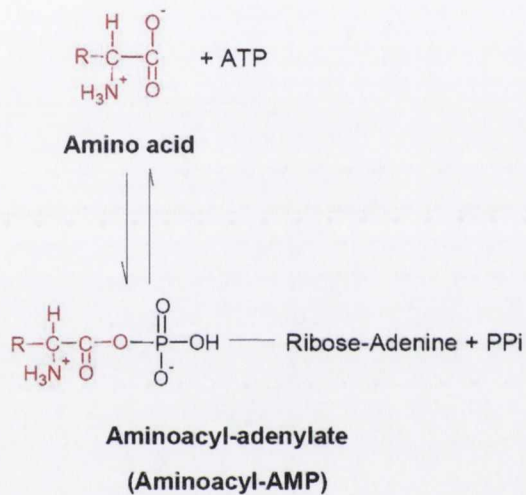


Figure 1.4 The secondary and tertiary structures of tRNA **A.** Three-dimensional representation of the L-shaped tRNA tertiary structure. **B.** Schematic representation of a “clover-leaf” tRNA secondary structure with the anticodon opposite to the amino acid attachment site. The amino acid is attached by an aminoacyl tRNA synthetase to the 3' end of the tRNA molecule.

A.



B.

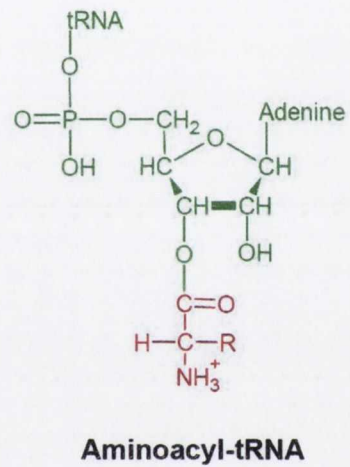


Figure 1.5 Formation of aminoacyl-tRNA **A.** Formation of aminoacyl-adenylate through the ‘activation’ of amino acid by reaction with ATP. **B.** Chemical structure of aminoacyl-tRNA (Adapted from Voet & Voet [11])

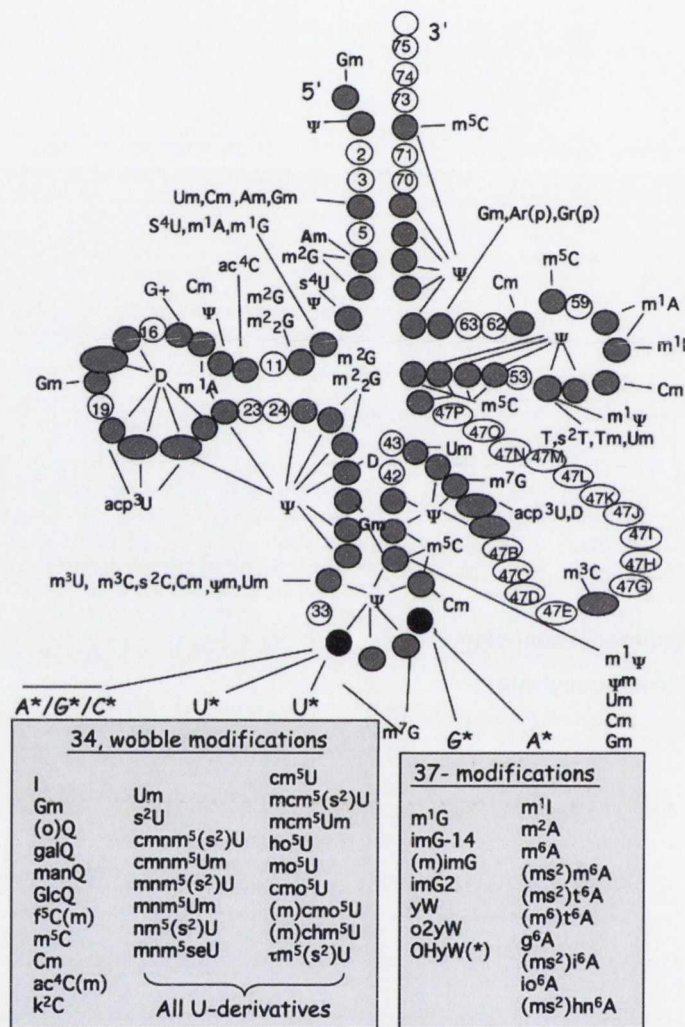
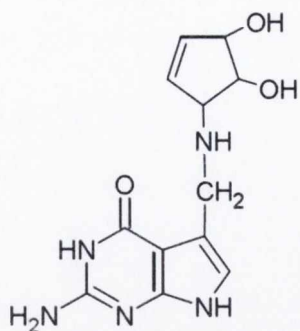


Figure 1.6 Schematic representation of secondary tRNA structure with identified nucleoside modifications. Ninety five post-translational modifications had been found so far in organisms studied. These modifications include methylation, hydroxymethylation, deamination, transglycosylation, acetylation, reduction, thiolation, oxidation and many more [10].

A.



B.

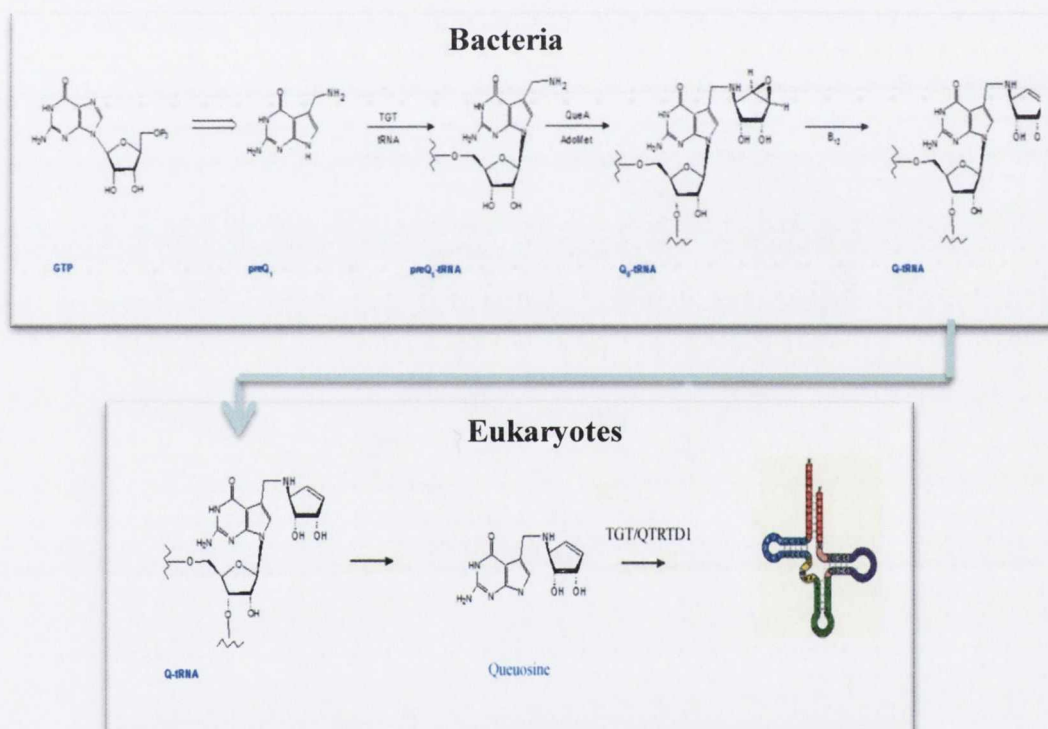


Figure 1.7 Structure of queuine and queuosine biosynthesis A. Chemical representation of the queuine molecule. B. Proposed mechanism for biosynthesis of queuosine in bacteria and eukaryotes. Abbreviations for the enzymes involved in queuosine formation are given in the main text.

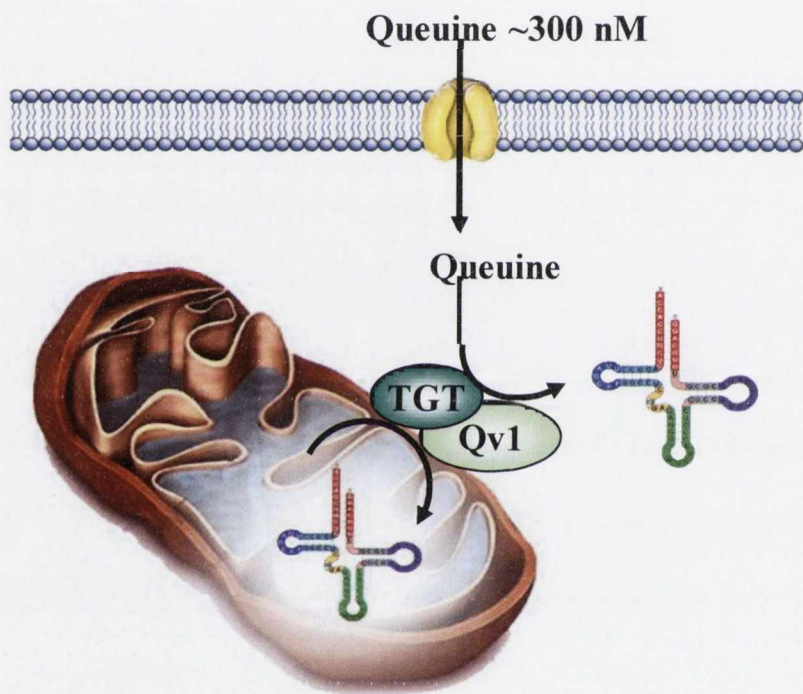


Figure 1.8 Proposed mechanism for queuosine formation in eukaryotic tRNA. Eukaryotes queuine is present in the serum at a concentration of approximately 300 nM. It is taken into the cells by unidentified transporters and inserted into both cytosolic and mitochondrial tRNA by a mitochondrial-localised heterodimeric transglycosylase complex consisting of TGT and a splice variant of QTRTD1, referred to as Qv1.

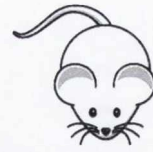


Table 1.1 The necessity of tyrosine in diet of germfree mice maintained on a chemically defined diet

	<i>Diet with tyrosine deaths/No. of mice</i>	<i>Diet w/o tyrosine deaths/No. of mice</i>
<i>Experiment 1</i>	0/18	14/14
<i>Experiment 2</i>	1/9	8/8

Note. Germfree mice were fed diet containing tyrosine for 62 days before withdrawal.

Table 1.2 Symptoms of Germfree mice maintained on a diet deficient in Tyrosine and Queuine

Symptoms of germfree mice – tyrosine/ - queuine

Squinting
Rough coat
Stiffness of neck and forelimbs
Lethary
Staggered walk
Huddling
Labored breathing
Seizures

Death

Note. The symptoms are listed in order in which they appeared

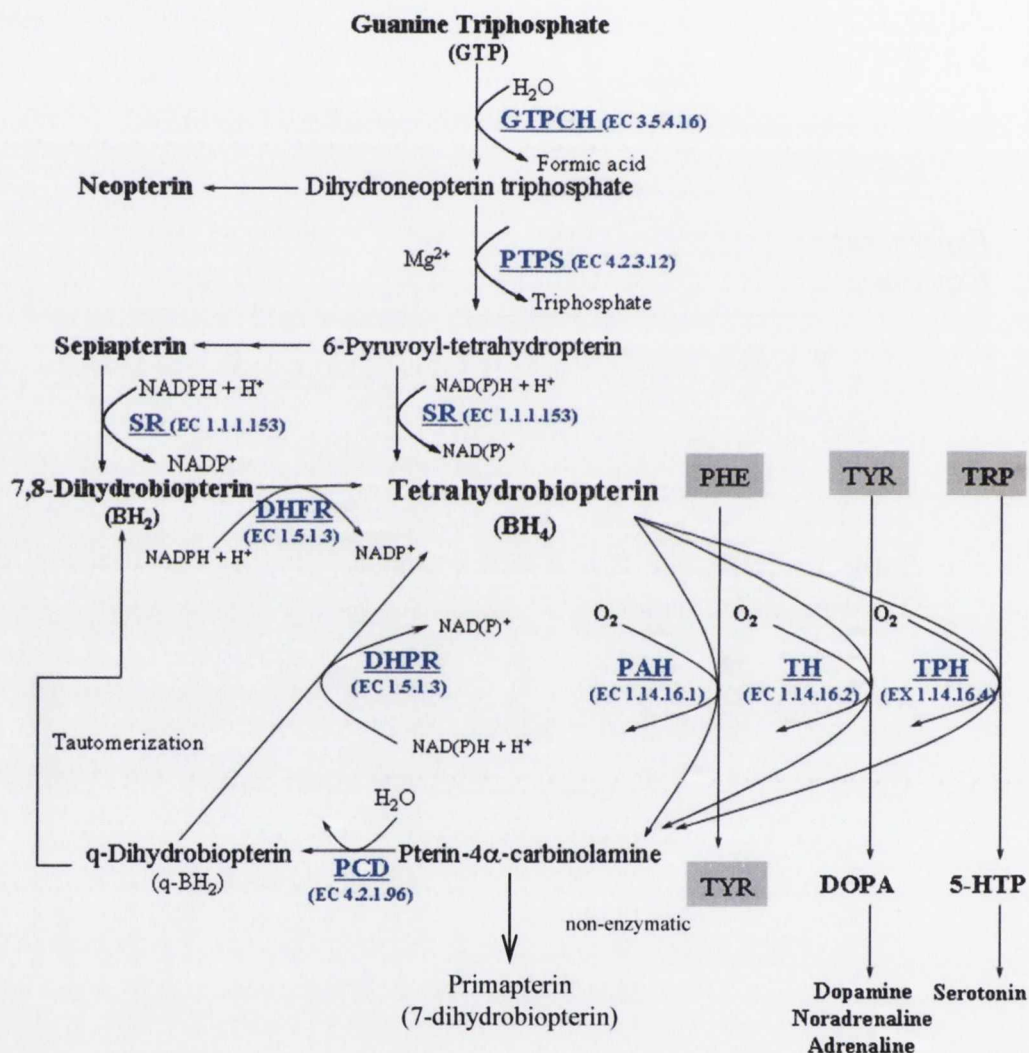


Figure 1.9 Aromatic amino acid hydroxylases and biosynthesis and regeneration of tetrahydrobiopterin. The tetrahydrobiopterin cofactor is used by phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) for the production of tyrosine and many vital neurotransmitter molecules as shown in the figure. Abbreviations for the individual enzymes are given in the main text.

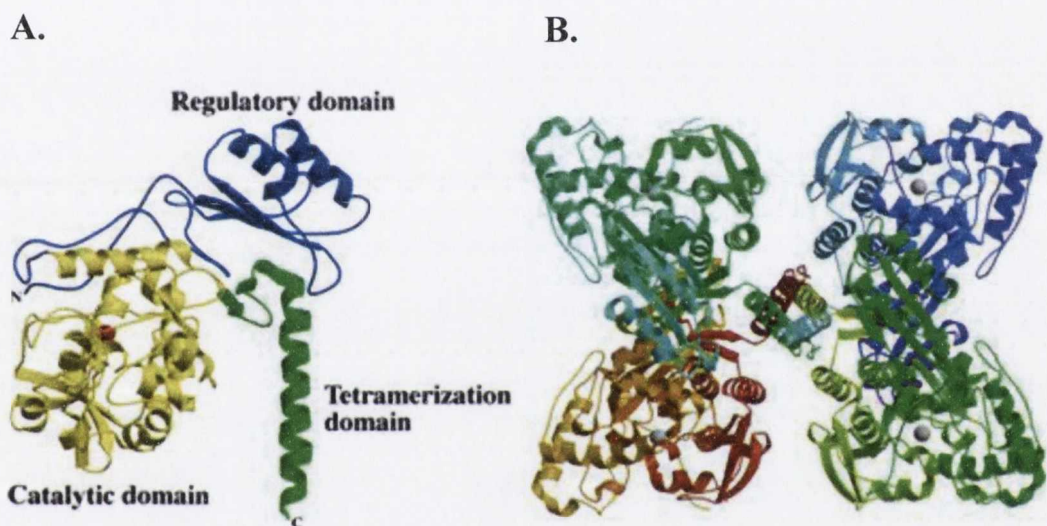


Figure 1.10 Three dimensional representation of PAH protein **A.** The overall structure of the full-length monomer and **B.** the model of the full-length tetramer. PAH consists of regulatory domain, containing an N-terminal autoregulatory sequence that is positioned over the active site, a catalytic domain that contains an active site iron and the substrate binding sites for BH₄ and phenylalanine, and a C-terminal oligermerization domain.

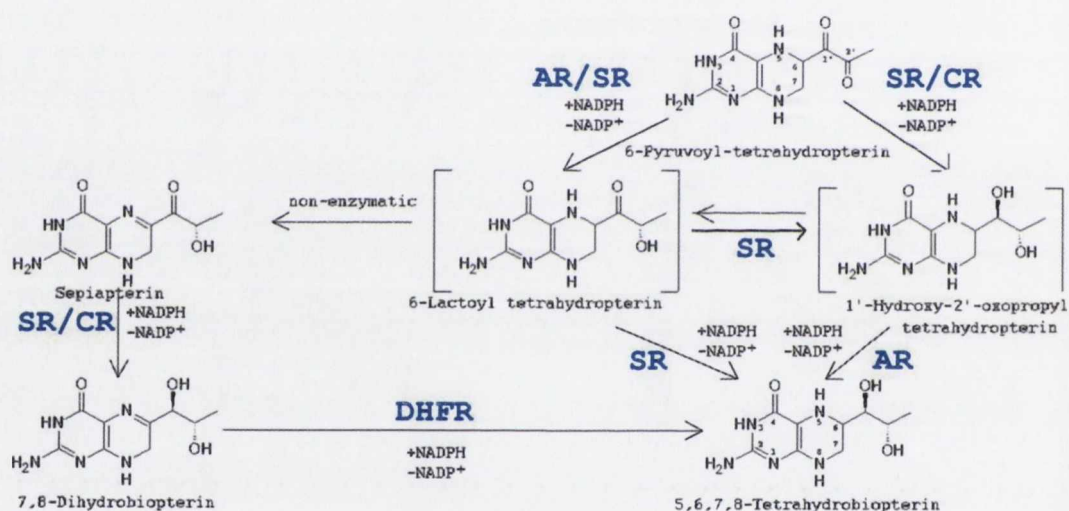
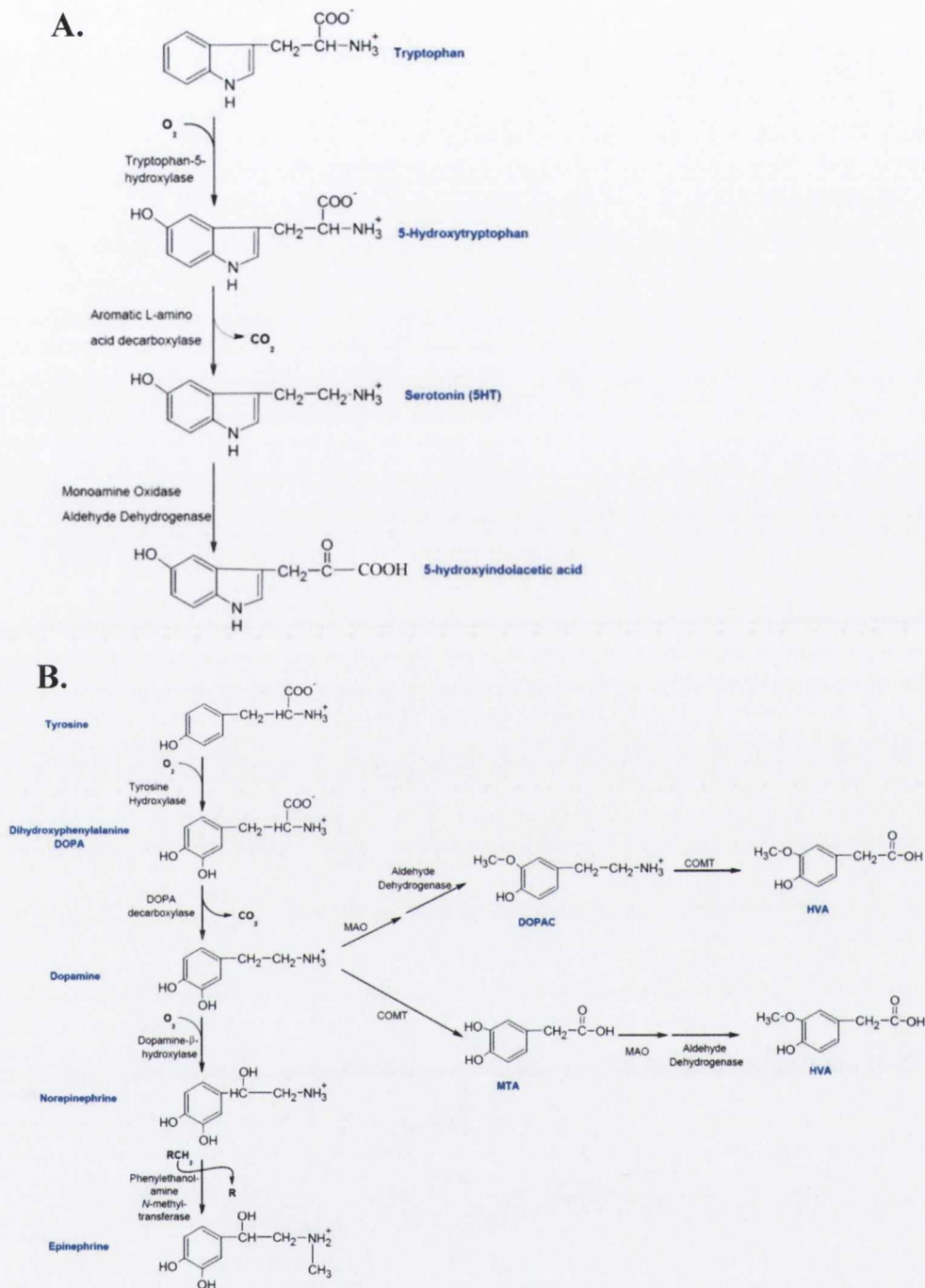
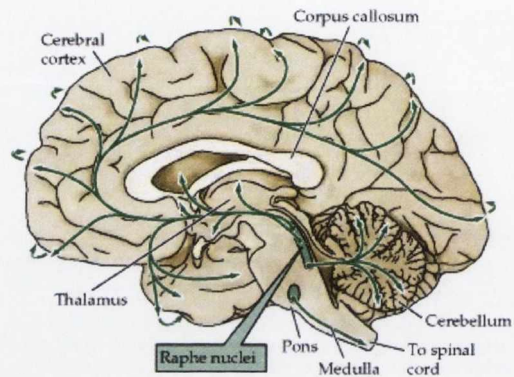


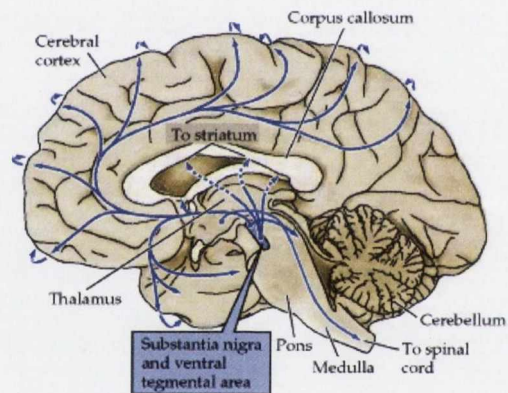
Figure 1.11 Salvage pathway of tetrahydrobiopterin. The tetrahydrobiopterin salvage pathway is carried out via sepiapterin. AR, aldose reductase; SR, sepiapterin reductase; CR, carbonyl reductase; DRFP, dihydrofolate reductase.



Serotonin



Dopamine



Norepinephrine

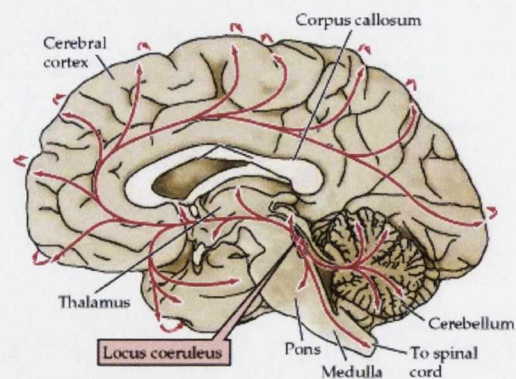


Figure 1.13 Distribution of biogenic amine neurotransmitters in the brain. Serotonin is located in the neurons of the raphe region within the brainstem, which have widespread projections to the forebrain. Dopamine is located several brain regions with the major dopamine-containing region being corpus striatum, which receives signals from substantia nigra. Norepinephrine is synthesized mainly by the sympathetic ganglia.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Materials used

Restriction enzymes and yeast tRNA were obtained from Roche Applied Science (West Sussex, UK). Bacterial alkaline phosphatase, dNTP mix, random priming labelling kit, 10x*Pfu*Turbo DNA Polymerase, Taq DNA Polymerase, T₄ DNA ligase, were obtained from Takara (Otsu, Shiga, Japan). Ultra-pure agarose, ampicillin, ascorbic acid, bentonite, boric acid, bovine serum albumin, bromophenol blue, CaCl₂, catalase, chloramphenicol, citric acid monohydrate, cyclohexamide, deuterated chloroform, deuterated methanol, deuterated water, dimethyl sulfoxide, direct load wide range DNA marker, Dithiothreitol, 1Kb DNA ladder, 0.5M EDTA solution, ferricyanide, formaldehyde, glycerol, glycine, Hepes, hydrochloric acid, imidazole, iodine, kanamycin, Kodak Biomax MS film, low range molecular weight marker, lysozyme, magnesium sulphate, 2-mercaptoethanol, 1M MgCl₂ solution, monoclonal anti-actin antibody, MOPS, octane-1-sulfonic acid, oxaloacetate, PBS, perchloric acid, L-phenylalanine, potassium acetate, potassium chloride, potassium hydroxide, potassium iodide, monobasic and dibasic potassium phosphate, potassium sodium tartate, protease inhibitor cocktail, rubidium chloride, sodium carbonate, sodium chloride, sodium dodecyl sulphate, sodium hydroxide, monobasic and dibasic sodium phosphate, sodium sulphate, sucrose, trichloroacetic acid, Trizma base, Tris-HCl, 1M Tris-HCl pH 7.5 solution, Tri sodium chloride, L-tyrosine, L-tyrosine disodium salt, urea were all purchased from Sigma-Aldrich Co. Ltd (Sigma-Aldrich, Ireland Ltd., Dublin, Ireland). Phenol-Chloroform, 1.5 RNase-free tubes, RNase-free water, RNALater, RNase Zap, Superase. In RNase inhibitor were purchased from Ambion (Austin, TX, USA). Superscript III RT and Ultrapure Phenol, TOP10 bacterial strain were obtained from Invitrogen (Carlsbad, California, USA). 30% Acrylamide/bisacrylamide 37:5:1, ammonium persulphate and Bradford reagent (5X) and TEMED solution were purchased from Bio-Rad (Hercules, California, USA).

Scintillation fluid and Absolve were purchased from National Diagnostics (Atlanta, Georgia, USA) and PerkinElmer (Watham, Massachusetts, USA), respectively. BL21(DE3) and BL21 RIL (DE3) bacterial strains were purchased from Novagen (Darmstadt, Germany). XL1-Blue bacterial strain was obtained from Stratagene (La Jolla, CA 92037, USA). Isopropylthio- β -S-galactoside (IPTG) was obtained from Calbiochem (Darmstadt, Germany). LB Broth and 2xYT medium were obtained from Chromatin (Dublin, Ireland) and Bacto Agar was from Difco (New Jersey, United States). Plasmids pET15b and pET21a were purchased from Novagen (Darmstadt, Germany). Enhanced chemiluminescence solutions, immobilon-P transfer membrane, Milli-Q Direct 3 water filtration system and the Millex GP 0.22 μ m filters were purchased from Millipore (Billerica, Massachusetts, USA). Horseradish peroxidase-labelled secondary antibody (goat anti-rabbit IgG), ECL mouse IgG (HRP-linked whole Ab (from sheep)), ECL rabbit IgG (HRP-linked whole Ab (from donkey)), pre-packed 1ml GSH and Nickel columns, Hybond N hybridization transfer membranes were from Amersham and Pharmacia (Buckinghamshire, UK). DMEM-high glucose medium, L-glutamine, Fetal Bovine Serum, Horse Serum, penicillin-streptomycin, sodium pyruvate, TrypLE Select solution, ultra pure buffer saturated phenol were obtained from BD Bioscience (San Jose, California, USA). The HPLC system was from Shimadzu (Kyoto, Japan). C18 ODS1 and Zorbax 300-SB C18 HPLC columns were from Agilent Technologies (Santa Clara, CA 95051, USA) and MTC (Eatontown, NJ 07724, USA) respectively. Acetic acid and HPLC grade solvents were obtained from Hazardous Materials facility in TCD (Dublin, Ireland). The Amylose resin was purchased from New England Biolabs (Ipswich, Massachusetts, USA). Whatman filter paper and DE52 were obtained from Whatman (Maidstone, UK). Trimethylsilyl propanoate (TSP) was kindly given by Dr. Kenneth Hun Mok. Tyrosine free chemically defined diet (AIN-76A purified diet) was obtained from Harlan Teklad, Custom Research Diets.

2.1.2 Sterilization of solutions and equipment

Solutions were sterilized at 121⁰C, 105Pa for 60 minutes in an autoclave. Heat sensitive solutions were filtered through a sterile filter (0.22 or 0.45 μ m pore size). Glass was autoclaved in the same manner as described before.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Culturing of hepatoma cell lines

During the course of this study, an adherent epithelial cell line, human HepG2, isolated from a liver biopsy of a male Caucasian, with a well differentiated hepatocellular carcinoma, was used. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were grown in DMEM medium, supplemented with 2mM L-glutamine, 0.1mg/ml penicillin/streptomycin, 1mM sodium pyruvate, and 10% Fetal Bovine Serum (FBS) or Horse Serum (HS), or in Serum Free Ultraculture Medium (supplemented with 2mM L-glutamine and 0.1mg/ml penicillin/streptomycin), with or without chemically synthesized queuine (a gift from Dr. Susumu Nishimura, Tsukuba University, Japan).

2.2.1.2 Queuine supplementation of serum free medium

A 100µM stock solution of chemically synthesized queuine was prepared in ultrapure water and stored at 4 °C. Serum free Ultraculture medium was made queuine sufficient by adding a final concentration of 300nM queuine.

2.2.1.3 Gelatinisation of cells culture flask

To assist the adherence of HepG2 cells when grown in DMEM medium supplemented with HS, gelatinization was performed. A stock of 0.1% bovine gelatin solution was prepared in sterile PBS and autoclaved. Addition of 3ml per 25cm² surface area was needed to coat the flask. Gelatinisation occurred after incubation at 37 °C for 1 hour or overnight. The gelatin solution was removed immediately prior to the addition of cells to the plate/flask.

2.2.1.4 Passage of cell lines

Cells were passaged upon reaching 80% confluence. Growth medium was removed from the cells, which were then washed once with the appropriate amount of PBS (1ml/1cm²). TrypLE Select solution, a non-animal alternative to porcine trypsin, was added to the cells (1.5ml/75cm² surface area) and detachment was induced by incubating the cells at 37 °C for approximately 5 minutes. Subsequently, fresh medium was added to the dish (4ml/75 cm² surface area) and a single cell suspension was achieved by gently pipetting the medium up and down several times. The cell pellet was collected by centrifugation at 1200xg for 5 minutes, and cells reseeded at a ratio of 1:3 to 1:6, depending upon the length of time needed before the cells were used for experiments.

2.2.1.5 Cell counting using a hemocytometer

Cell concentration was determined by counting their number within a defined area of known depth (volume). At least 10⁶ cells/ml were required for accurate counting. A hemocytometer was prepared by laying a clean coverslip over the grooves. A single-cells suspension was produced after cell detachment from the plate/flask. Using a pipette, 20µl was transferred to the edge of one side of the hemocytometer to fill one chamber. The slide was transferred to the microscope and a large square ruled by three lines was viewed. The total number of cells, inside the 25 major squares, was counted. Cells that overlapped the bottom and right border of squares were counted but those overlapping the top and left borders were ignored. The counting was repeated for the second chamber to give a total of 50 squares. The number of cells counted in 50 squares was added, divided by 50 and multiplied by 25 to give a number of cells in 1x10⁻⁴, and then multiplied by 10,000 to give the number of cells/ml.

2.2.1.6 Cryopreservation of cell lines

Cells were cultivated in T175 flasks until they reached 80% confluence. They were detached from the flask using TrypLE and centrifuged at 100 x g for 5 minutes. The cell pellet was resuspended in 12ml of fresh freezing mix (95% growth medium, 5% Ultrapure DMSO). Aliquots of 1ml, containing approximately 2x10⁶ cells, were dispensed into cryotubes. These were then placed in a cell freezing device which

contained isopropanol (Mr. Frosty TM; Nalgene) and transferred to -70 °C for 24 hours before being moved to a liquid nitrogen container for long-term storage.

2.2.1.7 Re-establishing cell lines from frozen stock

Cryotubes that had been maintained in liquid nitrogen, containing approximately 2×10^6 cells/ml, were thawed by partially submerging them in a 37 °C water bath. Once the ice-crystals had melted, the cells were placed in 12 ml of fresh medium and pelleted by centrifugation at 1200xg for 5 minutes. The pellet was resuspended in 12 ml of medium and plated onto a 10cm² cell culture dish.

2.2.2 *E.coli* strains and plasmids

2.2.2.1 *E.coli* strains

The genetic characteristics of the bacterial strains used are given in Table 2.1.

Table 2.1 Genetic description of bacterial strains

BL21 RIL (DE3) (Stratagene)	<i>E.coli</i> B F ⁻ dcm ompT hsdS (r _b ⁻ m _b ⁻) dcm ⁺ Tet ^r gal λ(DE3) endA The [argU ileY leyW Cam ^r)
BL21 DE3::TGT km _r (Stratagene)	<i>E.coli</i> B F ⁻ dcm ompT hsdS(r _b ⁻ m _b ⁻) gal λ(DE3)

2.2.2.2 Growth Media

Liquid LB and LB agar were routinely used to grow BL21 bacterial cells. However, for the production of recombinant *E.coli* TGT enzyme (Section 2.2.8.1) 2xYT broth was used. The method of preparation of these mediums can be found in Table 2.2.

Table 2.2 Composition of bacterial growth media

Media	Composition
Luria-Bertani broth (liquid LB)	10 g Bacto Tryptone, 5 g Yeast Extract, and 5 g NaCl in 1 litre of H ₂ O, and adjusted to pH 7.0 with NaOH
Luria-Bertani agar (LB agar)	Liquid LB containing 1/5% w/v agar
2xYT Broth	16 g Bacto Tryptone, 10 g Bacto Yeast Extract and 5 g NaCl in 1 litre of H ₂ O, and adjusted to pH 7.0 with NaOH

2.2.2.3 Plasmid vectors

The pET, pGEX and pMAL vectors were used to express recombinant proteins under the control of an isopropyl β-D-thiogalactoside (IPTG)-inducible T7 and *Tac* promoter, respectively.

Recombinant proteins generated from the pET15 and pET21a vectors contain a polyhistidine tag fused to the N-terminus. The pGEX vectors fuse a glutathione S-transferase (GST) from *Schistosoma japonicum* to the N-terminus of recombinantly expressed proteins. The pMAL vector fuses a 42kDa Maltose Binding Protein to the C-terminus of the expressed protein. Plasmid vectors and their uses are depicted in Table 2.3.

Table 2.3 Plasmid vectors and their uses

Vector	Use and properties
pET-15b (Novagen)	Bacterial expression, N-terminal His-tag
pET21a (Novagen)	Bacterial expression, C-terminal His-tag
pGEX-6P1 (Amersham)	Bacterial expression, N-terminal GST-tag
pMAL (New England Biolabs)	Bacterial expression, C-terminal MBP-tag

2.2.2.4 Antibiotics used for plasmid selection in *E.coli*

Antibiotics were prepared as depicted in Table 2.4. The solutions were filter-sterilized through 0.45µm syringe filter tips before the stock solutions being stored at -20 °C.

Table 2.4 Stock solutions of antibiotics and working dilutions

Antibiotic	Stock concentration	Working concentration
Ampicillin	100mg/ml (H ₂ O)	50-100µg/ml
Chloramphenicol	34mg/ml (ethanol)	34µg/ml
Kanamycin	100mg/ml (H ₂ O)	50-100µg/ml

2.2.2.5 Agarose gel electrophoresis and DNA extraction

DNA was routinely separated using agarose gel electrophoresis, essentially as described by Sambrook *et al.* (1989). An agarose gel was made up with 1 x TAE buffer (0.04 M Tris, 1.1% v/v acetic acid, 1 mM EDTA, pH 8.5), 1.5% agarose and 5µl ethidium bromide. DNA samples containing 100ng to 1µg of DNA mixed with 1/10 volume gel-loading dye (15% w/v ficoll; 0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol FF; 1 mM EDTA in MilliQ water) and resolved at 100 V for one hour with 1xTAE as running buffer. The desired bands were excised from the gel under minimum UV light exposure and purified using the Qiagen gel extraction kit. The DNA was eluted using 30µl of Nuclease Free Water.

2.2.2.6 Preparation of permanent plasmid stocks

E. Coli containing the appropriate plasmid were grown overnight in 3.5 ml of liquid LB broth containing selection antibiotic at 37 °C. In the morning a volume of 850µl of overnight culture was added to 150µl glycerol, vortexed and stored at -70 °C.

2.2.3 Mice genotyping

2.2.3.1 Isolation of genomic DNA from mouse ear punches

The small amount of tissue from one ear punch was adequate for genotyping mice. Ear punches were performed on 21-day old mice. Samples were placed in an eppendorf tube and used directly or stored at -20⁰C overnight.

Samples were each digested in 500µl of freshly prepared digestion buffer (1 x SSC (15 mM trisodium citrate, 0.15 M NaCl, pH 7-8), 1 mM EDTA, 20 mM Tris-HCl p H7, 1% SDS, 4µl Protein K per sample). The tubes were incubated at 55 ⁰C for 3 hours or overnight with gentle shaking. The extraction was carried out with an equal volume (500µl) of phenol:chloroform. Samples were mixed by inversion for 5 minutes. Samples were then centrifuged at 20,000 x g for 5 minutes. Using a tip with the end cut off the aqueous layer was removed to a new eppendorf. The DNA was precipitated by addition of an equal volume (500µl of isopropanol. Tube was inverted until DNA fibres became clearly visible. The DNA was pelleted by centrifugation at 5,900 x g for 30 seconds. The pellet was washed with 500µl of a 70% ethanol solution. The genomic DNA was resuspended in 100µl of water; the lid of the eppendorf was left open and heated at 65 ⁰C until all ethanol had evaporated. The concentration of samples was usually approximately 400µg/µl.

2.2.3.2 GoTaq screening

Specific primer sets were made to amplify the wild type and gene-targeted alleles.

Wild-Type allele primers (wta)

Name	Sequence (5'→ 3')
TGTFor	GAGGCCGGTGTGTGGATTCGATCTG
TGTRev3	CAGAGCATTCTGGATCTCCACCG

Genetrap insert (gti)

Name	Sequence (5'→ 3')
TGTFor	GAGGCCGGTGTGTGGATTCGATCTG
BgeoRev	TCTAGCCTCGAGGTCGACGGTATCG

HYG cassette primers (hyg)

Name	Sequence (5'→ 3')
inbgeofp1	GCACGCTGATTGAAGCAGAAGCCTGC
inbgeorp1	GGTCAGACGATTCATTGGCACCATGC

pGeo cassette primers (neo)

Name	Sequence (5'→ 3')
inhygfp1	AAGTTCGACAGCGTCTCCGACCTGATG
inhygrp1	CGCCATGTAGTGTATTGACCGATTCC

After isolation of genomic DNA from a mouse ear punch GoTaq was used to screen the samples as shown in Table 2.8 and 2.9. The PCR amplicons were run on a 2% agarose gel; 120 volts, ~40 min.

Table 2.8 PCR reaction mix

Component	Final Volume (μ l)	Final concentration
5 x GoTaq Flexi Buffer	5	1 x
MgCl ₂ Solution 25 mM	2	2 mM
Takara dNTP mix 2.5 mM each	4	0.2 mM each dNTP
Up-Primer (10 pmol/ μ l)	0.5	0.4 μ M
Down-Primer (10 pmol/ μ l)	0.5	0.4 μ M
Digested & purified mouse ear sample	1	N/A
H ₂ O	11.75	N/A
GoTaq polymerase (5 units/ μ l)	0.25	1.25 units

Table 2.9 PCR conditions

Wild-type allele (wta) and genetrap insert (gti)			B-geo (neo) and HYG (hyg) cassette		
Temperature (°C)	Time	} 35X	Temperature (°C)	Time	} 35X
95	5min		95	5min	
98	10sec		98	10sec	
68	30sec		65	30sec	
72	2 min		72	1 min	
72	8 min		72	8 min	
4	∞		4	∞	

2.2.4 Biochemical Protein Analysis

2.2.4.1 Protein Concentration Determination, Microplate Bradford Protein Assay

The Bradford assay is a colorimetric protein assay that is based on an absorbance shift in the dye Coomassie. Upon binding of coomassie dye to protein, the previously red form of coomassie reagent changes and stabilizes into coomassie blue dye. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Different concentrations of Bovine serum albumin, ranging from 50-500 µg/ml, were used as protein standards. A 10 µl volume of either protein sample or standard were added to the well of the 96-well plate. In quick succession 200 µl of 1:5 diluted Bradford reagent was added to each of the wells. The plate was incubated at room temperature for 5 minutes. The absorbances were then taken using a microplate reader (Spectra max 340PC).

2.2.4.2 Protein Concentration Determination, Markwell Assay

This protein determination assay is based on the method of Markwell *et al.* (1978). Bovine serum albumin was used as a standard protein. Reagent C was made by mixing 100 ml of reagent A (2.0% w/v sodium carbonate, 0.4% w/v sodium hydroxide, 0.16% w/v potassium sodium tartrate, 1.0% w/v SDS) and 1 ml of reagent B (4.0% w/v copper sulphate). The BSA (0-100 µg) or unknown protein in a volume of 1 ml, was mixed with reagent C (2 ml). After 10 minute incubation, 50% v/v Folin-Ciocalteu solution (0.2 ml) was added. The solutions were left to develop for 45 minutes and the absorbance values at 660 nm were determined using a Gilford 240 spectrophotometer. The absorbance values of the standards were fitted to a straight line and this was used to estimate the protein concentration of the unknown samples.

2.2.4.3 Isolation of total protein from cultured cells

To isolate the protein, mammalian protein extract reagent (M-PER) was used. M-PER extracts cytosolic and nuclear protein from cultured mammalian cells using a proprietary detergent in 25mM bicine buffer (pH7.6).

HepG2 cells were grown to a confluence of 80%. Growth medium was removed from the cells, which were then washed once with PBS. Appropriate amount of M-PER Reagent, protease (1µl/20mg) and 50x phosphatase (1µl/ml) inhibitors were added to the plate or to each plate well (see Table 2.10). The plate was gently shaken for 5 minutes and placed on ice. Cell lysate was collected and transferred to a microcentrifuge tube. Samples were centrifuged at 14,000 x g for 10 minutes to pellet the cell debris and supernatant was then transferred to a new tube for further analysis.

Bradford analysis was used to measure the protein concentration of each sample. Samples were prepared for SDS-PAGE Electrophoresis by standardising protein concentration to 1µg/µl with 2 x SDS-loading buffer. The loading buffer was always used at a 1:1 volume ratio or higher to protein extract. Samples were stored at -70 °C, if not used immediately.

Table 2.10 Suggested volume of M-PER Reagent to used for different sizes of standard culture plates

Plate Size/Surface Area	Volume of M-PER Reagent
100mm*	500-1,000 µl
60mm	250-500 µl
6-well plate	200-400 µl per well
24-well plate	100-200 µl per well
96-well plate	50-100 µl per well

*Cells grown in 100mm plates typically contain 10⁷ cells (50 mg) and yield ~3 mg total protein depending on cell type

2.2.4.4 SDS Polyacrylamide Gel Electrophoresis

Protein samples for electrophoresis were prepared by mixing the sample with an equal volume of 2 X SDS gel loading buffer (0.5M Tris-HCl pH 6.8, 4.4% w/v SDS, 20% v/v glycerol, 2% v/v 2-mercaptoethanol, and bromophenol blue (1%), made up in Milli-Q water). The mixture was then heated to 98 °C for 2 minutes and 30 seconds.

SDS-PAGE analysis was carried by the method of Laemmli (1970). Proteins were routinely separated on a 12% SDS polyacrylamide gel (see Table 2.11). The components

of the resolving gel were mixed in a 20 ml tube and poured in the gel mould and covered with a layer of isopropanol. When set, the isopropanol was removed and a piece of filter paper was used to remove excess water. A 12-well comb was then inserted into the mould and the stacking gel mixture added. Once the stacking gel was set the gasket and clips were removed and the gel was transferred to the gel apparatus (ATTO AE-6400 mini-gel system) containing gel running buffer (50 mM Tris, pH 8.3, 400 mM Glycine, 0.1% w/v SDS). More running buffer was added, once the gel was positioned in gel apparatus to cover the wells and each well was rinsed to remove excess acrylamide. A molecular marker was added to the first lane and samples were loaded into proceeding lanes. Electrophoresis was carried out at 35 mA per gel until the gel-tracking dye had reached the bottom of the gel.

Table 2.11 Components for SDS PAGE Gel

Components	12% Stacking Gel (ml)	5% Resolving Gel (ml)
H ₂ O	3.3	2.7
30% Acrylamide mix	4	0.67
1.5 M Tris pH 8.8	2.5	-
1.0 M Tris pH 6.8	-	0.5
10% SDS	0.1	0.04
10% Ammonium Persulfate	0.1	0.04
TEMED	0.004	0.004
Total Volume	10	4

2.2.4.5 Staining and De-staining Polyacrylamide Gels

After the tracking dye had reached the bottom of the gel it was removed from the electrophoresis apparatus and the gel was removed from between the gel plates. The stacking gel was discarded and the resolving gel was placed in a solution of 10% v/v acetic acid, 50% Methanol and 1.25% Coomassie Blue R for 10 minutes with gentle agitation. Destaining was carried out overnight in 1-% acetic acid and 30% Methanol with gentle agitation.

2.2.4.6 Western Blotting

After SDS-PAGE, the proteins were transferred electrophoretically from the gel onto Immobilon PVDF membrane using a 'semi-dry' transfer unit. The gel was removed from between the glass plates and immersed in 20 ml of Cathode Buffer (25 mM Tris, 40 mM Glycine, 10% Methanol, pH 9.4) for 15 minutes. The membrane was prepared by soaking in methanol for 15 seconds, nuclease free water for 2 minutes and Anode Buffer II (25 mM Tris, pH 10.4, 10% v/v methanol) for at least another 5 minutes. Six pieces of 3MM whatman paper were cut to the appropriate size in order to cover the membrane and gel. Two pieces of filter paper were soaked in Anode Buffer I (0.3 M Tris, pH 10.4, 10% v/v methanol), one piece in Anode Buffer II and three pieces in Cathode buffer for 2 minutes. The transfer system was set up as shown in Figure 2.2. The anode electrode plate was placed on a level bench top and two pieces of filter paper, which had been soaked in Anode Buffer I, were placed on the plate. Then the filter paper, which had been soaked in Anode Buffer II was placed on top of the first two sheets. The Immobilon PVDF membrane was then placed on top of the filter papers followed by the gel and then three pieces of filter paper soaked in Cathode Buffer were layered on top of the gel. The cathode electrode plate was then placed on top of the stack. Transfer was run for 1 hour at 120mA.

2.2.4.7 Antibody probing

After transfer the membrane was incubated overnight in TBST buffer (20 mM Tris HCl, pH7.4, 150 mM NaCl, 0.1% tween) containing 10% w/v dried skimmed milk (marvel). The membrane was incubated with antisera in TBST buffer containing 10% w/v dried skimmed milk (marvel). Dilutions of primary and secondary antisera used are shown in Table 2.12. The immobilised proteins samples were allowed incubate with the primary antibody for 1 hour with constant shaking. The membrane was washed 3 x 15 min with TBST to remove any unbound anti body. The membrane was placed in TBST buffer containing 10% w/v dried skimmed milk with a secondary antibody for 1 hour. The membrane was washed 3 x 15 min in TBST buffer. The antibody complex was visualised using enhanced chemiluminescence according to manufacturer's instructions.

Table 2.12 Primary and secondary antisera dilutions

Protein	1⁰Antisera Dilution	2⁰Antisera Dilution Donkey Anti-Rabbit
Phenylalanine Hydroxylase	1:5,000	1:20,000
tRNA:Guanine transglycosylase	1:2,000	1:20,000
Protein	1⁰Antisera Dilution	2⁰Antisera Dilution Anti-mouse from sheep
Tyrosine Hydroxylase	1:4,000	1:2,000
Actin	1:500	1:2,000

2.2.5 Tetrahydrobiopterin Measurements

2.2.5.1 Preparation of cellular extracts:

HepG2 cells were grown in Ultraculture medium (UC), UC medium with queuine or DMEM growth medium supplemented with 10% FBS in Corning Hyperflask Cell Culture Vessel. UC medium is a serum free medium and does not contain queuine. Supplementation of UC medium was made by the addition of 2 μ l/ml of 100 μ M chemically synthesized queuine dissolved in MilliQ water. Cells were harvested upon reaching 80% with the use of TrypLE solution. Cells were counted and cell pellet was collected by centrifugation at 100 x g for 5 minutes. The pellet was then resuspended in wash buffer (0.1 M Tris/HCl and 0.15 M KCl, pH 7.8) and the sample was centrifuged at 500 x g for 20 minutes. Two more washing steps were performed and after the last step the pellet was resuspended in 300 μ l of wash buffer. The intracellular content was extracted by repeated freeze-thawing by placing the samples in liquid nitrogen followed by heating by hand. The sample was centrifuged at 28,000 rpm for 15 minutes at 4°C and the clear supernatant was divided for acidic and basic treatments.

2.2.5.2 Preparation of liver cytosol fractions

Mice (wild-type, heterozygous and homozygous for TGT genetrapp insertion) were sacrificed by cervical dislocation and liver was removed. The liver sample was homogenized in cold homogenizing buffer (50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1mM EDTA, 1 mM DTT, Protease inhibitors) at a ratio of 5 μ l of homogenization buffer per mg of liver weight. The homogenate was then centrifuged at 15,000 x g for 20 min at 4 °C. The supernatant was removed and stored in -80 °C approximately 24 hours. Total protein content was determined by Microplate Bradford Protein Assay (Section 2.2.4.1).

2.2.5.3 Preparation of urine fractions

Urine samples were collected from mice (wild-type, heterozygous and homozygous) each day from 12 to 13 o'clock in the afternoon. Upon collection samples were kept at 4°C and

out of sunlight. Total protein content was determined by Microplate Bradford Protein Assay (Section 2.2.4.1).

2.2.5.4 Preparation of plasma fractions

Blood samples (approx. 150µl each) were collected from tail ventral caudal artery of mice (wild-type, heterozygous and homozygous) into eppendorfs containing K₂EDTA (36mg per L of blood) and 0.1% (w/v) DTT. Plasma was separated by centrifugation at 2,000 x g for 10 minutes at room temperature and frozen immediately at -80°C until analysis. Microplate Bradford Protein Assay was used to determine the total protein content of the samples (Section 2.2.4.1).

2.2.5.5 Preparation of brain samples

Mice (wild-type, heterozygous and homozygous for the TGT genetrap insertion) were sacrificed by cervical dislocation and brain tissue (Posterior Corte, Brain Stem and Spinal Cord) was removed. Brain samples were homogenized in cold extract buffer (50mmol/L Tris-Hcl of pH 7.4, 1 mmol/L DTT, 1mmol/L EDTA), and centrifuged at 16,000 x g for 15 minutes at 4°C. Protein was removed by adding 10µl of 1:1 mixture of 1.5 mol/L HClO₄ and 2mol/L H₃PO₄ to 90µl of extracts, followed by centrifugation at 16,000 x g. Upon collection brain samples were frozen at -80°C until analysis. Microplate Bradford Protein Assay was used to determine the total protein content of the samples (Section 2.2.4.1).

2.2.5.6 Measurement of Tetrahydrobiopterin levels

The BH₄ level was measured using HPLC analysis after iodine oxidation in acidic or alkaline conditions. Total biopterin represents the sum of BH₄, BH₂, and fully oxidized biopterin. Differential oxidation with iodine enables measurement of both total biopterin and BH₄. Under acidic conditions BH₄ and BH₂ are oxidized to biopterin, while under basic conditions only BH₂ is oxidized to biopterin and BH₄ undergoes side-chain cleavage to form pterin. The difference in biopterin content between two oxidations represents the actual BH₄ levels.

The separation was performed on a Spherisorb ODS1 C18 column (particle size 5 μm ; length x internal diameter = 250 x 4.6 mm; Waters) by as isocratic gradient in water with 5% v/v methanol at a flow rate of 0.6 ml/min. Pterins were detected by their native fluorescence (excitation wavelength 350 nm; emission wavelength 450 nm) by use of a 2475 fluorescence detector (Waters).

2.2.5.6.1 Preparation of samples for acidic and basic pH oxidation

Before oxidation it was made sure that samples oxidized were of the same concentration. Equal amount of clear HepG2 supernatant was used for acidic and basic oxidations. Liver cytosol fractions were made to a standard protein concentration by the addition of homogenization buffer (5 μl per mg of liver) thereby ensuring that the concentration of the liver fraction was the same for each genotype. Urine samples (6 μg each) were diluted with HPLC grade water by bringing the total volume to 190 μl . Equal concentrations of brain and plasma samples were taken (3mg/ml) by dilution to a required concentration with HPLC grade water.

2.2.5.6.2 Acidic pH oxidation

A 100 μl volume of sample was acidified by addition of 20 μl of 1M HCl and 50 μl of a 1% w/v iodine solution prepared in 2% potassium iodide. Samples were mixed and incubated for 1 hour in the dark at room temperature. The reaction was stopped by adding 10 μl of 5% w/v ascorbic acid and 20 μl water.

2.2.5.6.3 Basic pH oxidation

A 100 μl volume of sample 20 μl of 1M NaOH was added and oxidation was performed as described above. Samples were mixed and incubated for 1 hour in the dark at room temperature. The reaction was stopped by addition of 10 μl of 5% w/v ascorbic acid and 20 μl of 2 M HCl.

Oxidized samples were filtered through Millipore ultrafree-MC sterile centrifugal filter units (0.22 μm GV DURAPORE) by centrifugation at 5,000 g for 30 minutes. The

clear filtrate was injected onto the HPLC system. In the case of tetrahydrobiopterin analysis in brain samples ODS1 column was washed in between the runs with acidic buffer (0.5% methanol, 0.5% acetonitrile of pH 3.7 with acetic acid).

BH₄ levels were derived by subtracting biopterin levels under a basic oxidative state (biopterin + BH₂) from the levels under an acidic oxidative state (i.e. Total biopterin = biopterin + BH₂ + BH₄).

2.2.6 Expression and purification of Recombinant Proteins and Protein Activity Assays

2.2.6.1 Purification of Recombinant *E. Coli* TGT protein

Purification of TGT enzyme was necessary in order to carry out ^{14}C -guanine incorporation assays. Such assays allow the extent of queuine incorporation into the anticodon loop of tRNA to be evaluated.

E. coli BL21 DE3::TGT Km_r cell cultures were prepared in a 5 ml volume of 2YT broth with 100 $\mu\text{g/ml}$ of ampicillin and kanamycin. *E. coli* BL21 DE3::TGT Km_r cells were transformed with the *E. coli* TGT – pET15b plasmid, which carries an N-terminal His-Tag sequence. The transformed cells were cloned by plating on agar and a single colony selected for use. The cell cultures were then incubated overnight at 37 °C and 220 rpm in an orbital shaker. The 5 ml overnight cultures were transferred to 500 ml flasks of 2YT medium containing 100 $\mu\text{g/ml}$ of ampicillin and kanamycin. Cells were left to grow at 37 °C and 220 rpm. Absorbance values at 600 nm ($A_{600\text{nm}}$) were taken after 2 hour incubation. Cells were then grown until $A_{600\text{nm}}$ value was between 0.6 - 0.8 absorbance units. A 1 ml sample of uninduced cells was removed. The cells were pelleted and stored at -20°C. The cultures were cooled to 30°C and recombinant protein expression was induced with IPTG (final concentration of 0.5 mM). The cells were left at 30°C overnight with shaking at 220 rpm. The next morning, a 1 ml sample of induced cells was removed, pelleted and stored at -20°C. The cell cultures were centrifuged at 7,000 rpm for 6 minutes and stored at -20°C until required. Cells were resuspended in 60 ml of column wash buffer (5 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, 10% glycerol, pH 7.4), containing protease inhibitor (1 μl /20mg). To weaken the cell wall, 1 ml of lysozyme (10 mg/ml) was then added to each tube and cells were left to incubate on ice for 1 hour. After the incubation, cells were sonicated for 3 minutes with 1 second bursts using a Brandson Sonifier 250. The suspension was then centrifuged in SS34 tubes at 15,000 rpm for 40 minutes at 4 °C to remove cell debris. The supernatant was filtered through a 0.45 μm filter. A sample of supernatant was taken for SDS-PAGE electrophoresis and the rest of the supernatant was loaded onto a pre-packed 1 ml Nickel column at a flow rate of 1 ml/min. Once the supernatant was loaded onto the column, the column was washed with

15 ml of column wash buffer, 30 ml of 30 mM Imidazole buffer (30 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, 10% glycerol, pH 7.4), 5 ml of 50 mM Imidazole buffer (50 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, 10% glycerol pH 7.4) and 1 ml of 80 mM Imidazole buffer (80 mM Imidazole, 25 mM NaCl, 20 mM Tris-HCl, 10% glycerol, pH 7.4). The protein was eluted from the column using 5 ml of 250 mM Imidazole buffer (250 mM Imidazole, 25 mM NaCl, 20 mM Tris-HCl, 10% glycerol, pH 7.4). 1 ml fractions of the eluted proteins were collected.

Each fraction was checked with Bradford reagent to see which fractions contain protein (10 μ l of sample and 200 μ l of Bradford reagent). To remove Imidazole used to elute the protein 2.5 ml of eluted protein was then loaded onto a pre-equilibrated pd-10 column with 25 ml of wash buffer with no Imidazole. The 2.5 ml was allowed to enter the packed bed completely before any addition of buffer for elution. 3.5 ml of the wash buffer without Imidazole was then used to elute the enzyme from the column. The final enzyme concentration was determined with the use of Bradford assay, the purity was checked by SDS-PAGE, the sample was made up to 50% glycerol and stored at -20°C.

2.2.6.2 tRNA isolation from HepG2 cells

HepG2 cells were grown on Corning Hyperflasks in DMEM supplemented with 10% FBS or HS, or in ultraculture medium with or without added queueine (300nM final concentration). Once confluent, the cells were harvested by the use of TrypLE solution and rinsed briefly in ice-cold PBS. Before the centrifugation of the cells, the weight of the 50 ml tube was determined. Cells were then centrifuged at 1,200 x g for 5 minutes at 4°C and the weight of the pellet was determined. Cells were then stored in RNA-Later solution at -80°C until needed. HepG2 cells in RNA-Later were thawed on the bench and pelleted by centrifugation at 4,000 rpm for 10 minutes. The pellet was resuspended in 50 ml of the NTEB buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM Na₂EDTA & 0.4 mg/ml bentonite) and transferred into a GSA pot. A 50 ml volume of fresh 80% phenol was added to the solution and shaken vigorously at 4°C for at least 30 minutes. The sample was centrifuged in a GSA rotor at 11,000 x g for 15 minutes and the aqueous (upper) phase was carefully transferred to an RNase free tube. Solid potassium acetate (1.5 g) was added and the nucleic acids were precipitated with the addition of ice cold

125 ml absolute ethanol and incubated at -20°C for 4 hours or overnight. Precipitate was collected by centrifugation in a GSA rotor at $11,000 \times g$ for 15 minutes and dissolved in 10 ml of 50 mM NaCl, 10 mM MgCl_2 , 1 mM Na_2EDTA & 0.4 mg/ml bentonite, with shaking. 2.5 ml of 5 M NaCl was added to sample and placed on ice for 2 hours. After 2 hours the sample was centrifuge at $11,200 \times g$ for 15 minutes at 4°C , and the supernatant collected and stored on ice. The precipitate that formed during centrifugation was washed with 2.5 ml of 1 M NaCl containing 10 mM MgCl_2 , 1 mM Na_2EDTA . The 12.5 ml of supernatant and the 2.5 ml of washings were combined and mixed with 50 ml of 10 mM MgCl_2 & 1 mM Na_2EDTA . The crude tRNA sample was directly applied to the pre-prepared 15 ml DEAE-cellulose column at a flow-rate of 1.0 ml/minute. The column was washed with 10 bed volumes of equilibration salt solution (250 mM NaCl, 10 mM MgCl_2 , 1 mM Na_2EDTA). The tRNA was eluted in one step from the column with a solution of high-salt buffer (0.7 M NaCl, 10 mM MgCl_2 , 1 mM EDTA). Samples with an absorbance at 260 nm were pooled and adjusted to be in 300 mM Tris-HCl (pH 8.0). To deacylate the tRNA pooled samples were incubated at 35°C for 30 minutes. Ice cold 2.5 x volumes of absolute ethanol was added to the sample and after standing at -20°C overnight the tRNA precipitate was collected by centrifugation at $11,200 \times g$ for 15 minutes. The tRNA sample was re-dissolved in nuclease free water and the concentration of the sample was determined by taking the absorbance at 260 nm. The tRNA sample was stored at -70°C until required.

2.2.6.3 TGT Activity Assay in HepG2 cells

This assay is based on the ability of *E.Coli* TGT to insert $[8-^{14}\text{C}]$ guanine into tRNA that is not modified by queuine. Therefore, a low level of $[8-^{14}\text{C}]$ guanine incorporation signifies that tRNA is highly modified by the queuine. Before $[8-^{14}\text{C}]$ guanine (0.1mCi/ml; 55mCi/mmol) is used in a tRNA incorporation reaction it must be neutralised by the addition of an equal volume of 0.1 M NaOH, as the $[8-^{14}\text{C}]$ guanine comes supplied in 0.1 M HCl. Each of the components was added in the order shown in Table 2.13.

Table 2.13 Reaction mixture, order of components

Reagents	Stock	Take (μl)
50mM Tris-HCl, pH7.5	1M	2.5
20mM NaCl	1M	1
5mM MgCl ₂	250mM	1
2mM DTT	100mM	1
Water	-----	39.92
RNAase inhibitor	-----	0.25
tRNA (2mg/ml)	2 au/μl or 100μg/μl	1
Enzyme (2 μg/μl)	-----	1.33
¹⁴ C-guanine	-----	2

The reaction was incubated at 37⁰C for 2 hours. To stop the reaction, 150μl of Phenol:Chloroform (pH 4.5) solution was added. The sample was then mixed and centrifuged at 13,200 rpm for 2 minutes at 4⁰C. The upper (aqueous) layer was removed and loaded onto a 1 ml DE52 spin column. The sample was spun at 800 rpm for 30 seconds and the flow through was collected and loaded onto a column again. This step was repeated 5 times. The column was then washed with 2.5 ml of wash buffer (0.02 M Tris-HCl pH7.5, 0.01 M MgCl₂ and 0.2M NaCl) and the flow-through was collected. The free ¹⁴C-guanine was counted by scintillation counting. 1 ml of elution buffer (0.02 M Tris-HCL pH7.5, 0.01 M MgCl₂ and 1 M NaCl) was added to the column and it was left to incubate for 1 minute. The tRNA was then eluted by centrifugation. The radioactivity was then counted by scintillation counting. The eluates were transferred to 5 ml plastic vials containing appropriate amounts of scintillant (Ecoscint A) and the ¹⁴C dpms were determined using the scintillation counter set to Protocol 2.

2.2.6.4 Purification of Recombinant PAH

Eight starting cultures, prepared in the volume of 5 ml of LB broth with 100 µg/ml of ampicillin, were inoculated with *E.coli* BL21 DE3 cells transformed with the *E.coli* pMalE plasmid containing mouse phenylalanine hydroxylase (cDNA). This plasmid produces recombinant protein carrying a C-terminal MBP-Tag sequence. The transformed cells were cloned by plating on agar and a single colony selected for use. The cell cultures were then incubated overnight at 37°C and 220 rpm in the orbital shaker. The 5 ml overnight cultures were transferred to 500 ml flasks of LB medium containing 100 µg/ml of ampicillin. Cells were left to grow at 37°C and 220 rpm. Absorbance values at 600 nm (A_{600}) were taken after 2 hour incubation. Cells were then grown until $A_{600\text{nm}}$ value was between 0.6 - 0.8. A 1 ml sample of uninduced cells was removed; the cells were pelleted and stored at -20°C. The cells were cooled to 30 °C and recombinant protein expression was induced with IPTG (final concentration of 0.5 mM). The cells were left to at 30 °C for 4 hours with constant shaking at 220 rpm. A 1 ml sample of induced cells was then removed; cells were centrifuged at 7,000 rpm for 6 minutes and stored at -20 °C until required. Cells were resuspended in 60 ml of MBP column wash buffer (50 mM Tris-HCl, 100 mM NaCl, 10% glycerol), containing protease inhibitor. To weaken the cell wall, 1 ml of lysozyme (10 mg/ml) was then added to each tube and cells were left to incubate on ice for 1 hour. After the incubation cells were sonicated for 3 minutes with 1 second bursts using Brandson Sonifier 250. The suspension was then centrifuged in SS34 tubes at 15,000 rpm for 40 minutes at 4°C to remove cell debris. The supernatant was filtered through a 0.45 µm filter. A sample of supernatant was taken for SDS-PAGE electrophoresis and the rest of the supernatant was loaded onto a 1 ml MBP-column, pre-equilibrated with 20 ml of MBP-column wash buffer, at a flow rate of 1 ml/min. Once the supernatant was loaded onto the column, the column was washed with MBP-column wash buffer until A_{280} has decreased to 0.02. The protein was eluted from the column with MBP-column elution buffer (50 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 10 mM Maltose). 1 ml fractions of the eluted proteins were collected.

Concentration of each fraction was determined with Bradford assay and the sample purity was analysed by SDS-PAGE. The fractions were stored at -70°C.

2.2.6.4.1 Kinetic Analysis of Recombinant PAH Enzyme

PAH activity assay was based on the measurement of tyrosine production from phenylalanine. Tyrosine production can be measured by obtaining initial rates of reaction on a spectrophotometer set at 275 nm. The reaction mixture contained 0.1 M Potassium Phosphate, pH 6.8, 65 µg of catalase (made up in potassium phosphate buffer), 1 mM phenylalanine (stock prepared in 200 mM Tris-HCl buffer, pH 7.2 at 25 °C), 35 µg of PAH enzyme, 6 mM DTT and 6(R)-BH₄ with concentrations ranging from 0 to 100 µM. The baseline for the reaction was set by blanking the spectrophotometer with a cuvette containing all reaction components except phenylalanine. To activate the PAH enzyme, the potassium phosphate buffer, catalase, phenylalanine and enzyme sample were allowed to incubate for 3 minutes at 25 °C. DTT and 6R-BH₄ were added to start the reaction and the product formation was observed at 275 nm.

2.2.6.4.2 Kinetic analysis to study the effect of Queuine on recombinant PAH activity

Studies were performed with three different concentrations of Queuine (100nM, 300nM and 10µM) and tRNA with or without queuosine modification to determine if they influence tyrosine production by PAH. The reaction mixture contained 0.1 M Potassium Phosphate, pH 6.8, 65 µg of catalase (made up in potassium phosphate buffer), 1 mM phenylalanine (stock prepared in 200 mM Tris-HCl buffer, pH 7.2 at 25 °C), 35 µg of PAH enzyme, 0-10 µM Queuine, 6 mM DTT and 6(R)-BH₄ with concentrations ranging from 0 to 100 µM. The baseline for the reaction was set by blanking the spectrophotometer with a cuvette containing all reaction components except phenylalanine. To activate the PAH enzyme, the potassium phosphate buffer, catalase, phenylalanine and enzyme sample were allowed to incubate for 3 minutes at 25 °C. DTT and 6R-BH₄ were added to start the reaction and the product formation was observed at 275 nm.

2.2.4.6.3 Kinetic analysis of mouse PAH enzyme

Mice (wild-type, heterozygous and homozygous for TGT genetrapp insertion) were sacrificed by cervical dislocation and liver was removed. The liver sample was homogenized in cold homogenizing buffer (50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1mM EDTA, 1 mM DTT, Protease inhibitors) and total protein content was determined by Microplate Bradford Protein Assay (Section 2.2.4.1).

PAH activity assay was based on the measurement of tyrosine production from phenylalanine. Tyrosine production can be measured by obtaining initial rates of reaction on a spectrophotometer set at 275 nm. The reaction mixture contained 0.1 M Potassium Phosphate, pH 6.8, 65 µg of catalase (made up in potassium phosphate buffer), 1 mM phenylalanine (stock prepared in 200 mM Tris-HCl buffer, pH 7.2 at 25 °C), 50 µg of mouse liver homogenate, 6 mM DTT and 6(R)-BH₄ 100 µM. The baseline for the reaction was set by blanking the spectrophotometer with a cuvette containing all reaction components except phenylalanine. To activate the PAH enzyme, the potassium phosphate buffer, catalase, phenylalanine and enzyme sample were allowed to incubate for 3 minutes at 25 °C. DTT and 6R-BH₄ were added to start the reaction and the product formation was observed at 275 nm. It has to be noted that this method was not the best for accurate determination of PAH activity as this experiment was carried out in crude extracts. It might be useful in any future assays of crude fractions to use the assay linked to the reduction of NADP by measuring PAH at 340 nm in the presence of O₂, phenylalanine, catalytic amounts of dihydrobiopterin and substrate amounts of NADP. Alternatively, the consumption of oxygen could be measured in an oxygen electrode.

2.2.6.4.4 *In vitro* activity assay of PAH enzyme

Mice (wild-type, heterozygous and homozygous for TGT genetrapp insertion) were sacrificed by cervical dislocation and liver was removed. The liver sample was then homogenized in 5 ml homogenization tube. The resultant mix was centrifuged at 100,000 g in ultracentrifuge for 60 minutes at 4°C. Microplate Bradford Protein Assay was used to determine the total protein content of the samples (Section 2.2.4.1).

The reaction mixture (Total volume 0.8µl) contained; 0.2M KCl, 40mM K₂HPO₄, 3.4µg catalase, 0.2mM phenylalanine, 33nCi C¹⁴phenylalanine and Crude extract (19µl).

To start the reaction (6R)-5,6,7,8- BH₄ dihydrochloride (100μM) and DTT (4mM) were added to the mixture (0.2μl). Reaction was incubated for different time intervals (0, 5, 10, 20 and 60 minutes) and stopped by adding excess unlabelled phenylalanine and tyrosine with 1/10th volume of unmarked solution containing 0.05 M phenylalanine and 0.05 M tyrosine (0.2μl). Proteins were precipitated with 1 in 4 dilutions with ice-cold 5% (w/v) TCA (trichloroacetic acid) solution and pelleted by centrifugation for 5 minutes at 12,000 g. Supernatant was removed and spotted onto Thin Layer Chromatography (TLC) plates pre-coated with silica gel 60. TLC separation was then carried out by placing TLC plates in mobile phase (chloroform:methanol:ammonia (55:35:10)) which was poured in the container at 1 cm depth, in the fume hood a couple of hours before analysis in order to equilibrate. The separation was stopped when solvent was 2 inches from front and excess solvent was removed by placing TLC plate on a hot plate at 60°C. Autoradiography (Kodak XAR film) allowed visualisation of proteins.

2.2.6.4.5 *In vivo* assay of intracellular tyrosine levels in HepG2 cells

HepG2 cells were grown to 80% confluence in a 24-well plate in ultraculture medium, ultraculture medium supplemented with queueine (300nM) and in DMEM supplemented with FBS. Intracellular levels of tyrosine and phenylalanine were depleted by washing the cells with PBS, followed by a 1 hour incubation in ultraculture medium that did not contain tyrosine or phenylalanine (customized synthesis) and supplemented with cyclohexamide (20μg/ml; a protein synthesis inhibitor) with constant shaking at 70 rpm. In order to perform tyrosine measurements, cells were washed with PBS and incubated in KRHL medium (120 mM NaCl, 4.8 mM KCl, 10 mM D-glucose, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 2.5 mM Hepes, pH 7) containing 20 μM, 60 μM and 100 μM L-[2,6-³H]-Phenylalanine (40-60 Ci/mmol, 1 mCi/ml) with 20 μg/ml cyclohexamide for 30 minutes with constant shaking at 70rpm. The experiment was set up in duplicate for each of the medium conditions. The cells from one well were used to determine cell protein content. Cells were solubilised with mammalian protein extract solution (M-PER) and protein concentration was determined by Microplate Bradford Protein assay (See section 2.2.5.1). After 30 minutes incubation, the medium was removed and placed on dry ice for further analysis. Cells were rapidly washed with ice cold PBS, to prevent loss of counts. Cells

were then lysed with 100µl per each well ice-cold 10% TCA and scraped from the dish with a rubber policeman cell scraper. Lysate was centrifuged at 16,000 x g for 15 minutes and the supernatant was removed and stored at -70 °C until analysed.

Intracellular levels of tyrosine were measured by HPLC on a Zorbax 300SB-C18 column. The C18 column was equilibrated with Mobile Phase Buffer (100 mM sodium phosphate pH 1.9, 300 µM octyl sodium sulphate, 500 µM of EDTA and 6% HPLC grade Methanol. Samples of cell extracts (50µl) were spiked with cold tyrosine (0.1 mM) and phenylalanine (0.5 mM) and run on C18 column at 1 ml/min. Amino acids were detected by fluorescence with an excitation wavelength between 258 nm and 288 nm. Fractions (1 ml) were collected and radiolabelled tyrosine and phenylalanine were detected by scintillation counting.

2.2.6.4.6 *In vivo* assay of phenylalanine to tyrosine conversion in mice

Adult (2months old) wild-type, heterozygous and homozygous Filial3 (F3) C57BL/6J::129S4/SvJaeSor mice were used in this experiment. L-Phenylalanine (40 mg/ml) was dissolved in a solution of 0.9% Saline and 7.5 mM NaOH and injected intraperitoneally at 1mg phenylalanine per gram body weight.

At different time points blood was collected from tail caudal artery in K₂EDTA tubes and promptly centrifuged at 2,000g for 10 minutes at 4 °C. The plasma was removed and stored on ice until analysis.

Plasma was then deproteinized. A measured volume of plasma and 9 volumes of 1.11 M HClO₄ were mixed in an eppendorf. After 10 minutes the suspension was centrifuged for 6 minutes at 16,000 x g at 4 °C. A measured aliquot of supernatant was then treated with 0.091 volumes of 10 M KOH; incubated on ice for 10 minutes and centrifuged for 6 minutes at 16,100 x g at 4 °C. The supernatant was then filtered in Millipore ultrafree-MC sterile centrifugal filter units (0.22µm GV DURAPORE) by centrifugation for 1 min at 16,100 x g at 4 °C. The clear filtrate was stored on ice until analysis.

Separations were done at an ambient temperature on a Zorbax 300SB-C18 column. The C18 column was equilibrated with Mobile Phase Buffer (100 mM sodium phosphate pH 1.9, 300 µM octyl sodium sulphate, 500 µM of EDTA and 6% HPLC grade Methanol. Each sample was 65 µl per run, the pH of which was <7. The HPLC flow rate was set at 1

ml/min. Tyrosine and phenylalanine were detected by fluorescence with an excitation wavelength of 258 nm and an emission wavelength of 288 nm and the levels were quantified by determining area under the peaks using an integration package on the EZStart 7.3SPI Chromatography Software (Shimadzu).

2.2.6.5 Spectrophotometric Activity Assay of DHFR in Mouse Liver

Liver was homogenized on ice with 1g of liver: 3 ml of homogenization buffer (0.2M Tris-HCl, pH 7.6 at 4°C containing 10mM DTT and protease inhibitors). The homogenate was centrifuged at 100,000 x g for 60 minutes at 4°C, and supernatant was removed and stored at -80°C until analysis.

The standard spectrophotometric assay was used to measure the DHFR activity. Reactions were run in a water-jacketed cuvettes with the temperature maintained at 27°C, and NADPH plus 7,8-dihydrofolate consumption was monitored at 340nm with spectrophotometer. Formation of the tetrahydrofolate product was calculated using a millimolar extinction coefficient of 12.3mM⁻¹cm⁻¹ with 7,8-dihydrofolate as a substrate.

Reactions were run in 0.1M Tris-HCl, pH 7.4 at 27°C, 0.1mM NADPH, 50mM 2-mercaptoethanol, and 60μM 7,8-dihydrofolate and liver extract (100μg) as the source of DHFR. All components of the reaction mixture except substrate were temperature equilibrated for 5 minutes before initiation of reaction with 7,8-dihydrofolate. For the spectrophotometric assay the reaction was monitored during this time to observe any baseline rate due to non-specific oxidation of NADPH.

2.2.7 Mice Brain Analysis

2.2.7.1 Biogenic Amines Analysis in Brain samples from mice

2.2.7.1.1 Preparation of Brain Samples

In the first experiment 6-8 weeks old wild-type, heterozygous and homozygous (C57BL/6J::129S4 SvJaeSor) mice (6 of each genotype) of both genders were taken for biogenic amines analysis. Animals were housed 2-4 animals per cage and all were allowed free access to food and water. These animals were sacrificed by cervical dislocation and brain was extracted and four parts were dissected: forebrain, cerebellum, limbic brain and brain stem.

In the second experiment 8 mice of each genotype (four female and four male 8-10 weeks old mice) were used for biogenic amines analysis. These animals were also housed 2-4 animals per cage but were allowed free access to chemically defined food (Tyrosine free diet, TD-09362, Harlan Teklad, Custom Research Diets) and water containing sufficient amount of tyrosine (0.45g/L). These were sacrificed by cervical dislocation and brain was extracted and dissected into 6 subsections: hypothalamus, frontal cortex, striatum, hippocampus, cerebellum and the rest of the brain minus brainstem and posterior cortex which were retained for BH₄ analysis.

Each part of the brain was weighed and placed into buffer (mobile phase) containing 5 ng/20µl of N-methyl-5HT as an internal standard. The volume of the buffer was adjusted to bring the concentration of samples to 100 mg/ml. Samples were sonicated and centrifuged at 16,100 x g for 20 minutes at 4 °C. Supernatant was filtered through 0.45 µm filters and stored at -20 °C for biochemical analysis.

2.2.7.1.2 HPLC analysis

Samples were analysed using an automated high performance liquid chromatography (HPLC) – electrochemical detection system (Shimadzu). The HPLC method used was based on that of Seyfried et al (1986)[138] The mobile phase contained 0.1 M Citric acid monohydrate, 0.1M Sodium dihydrogen phosphate, 1.4 M Octane-1-Sulfonic Acid), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 9% methanol. The pH of the mobile

phase was adjusted to pH 2.8 with 4 M NaOH. The low pH is necessary to ensure retardation of acidic compounds and to provide an optimal concentration of the ion pairing reagent octane-1-sulfonic acid in order to manipulate retention times of the amines in the column. Retention times varied between 5 – 50 minutes on an LI Chrosorb RP-18 column. The flow rate of the mobile phase through the HPLC system was 1 ml/min at a pressure of approximately 200 bar. The column oven was maintained at 30 °C. Standards (NA, L-DOPA DOPAC, DA, 5-HIAA, 5-HTP, HVA, 5-HT, N-Methyl-5HT) were purchased from Sigma.

2.2.7.2 Behavioural Studies on mice

2.2.7.2.1 Subjects

Wild-Type, Heterozygous and Homozygous mice (C57BL/6J::129S4 SvJaeSor) for TGT genetrap insertion (6-8 weeks old) were used for behavioural analysis. Mice, 8 per each genotype (four females and four males) were housed 2-4 animals per cage and all were allowed free access to chemically defined food (Tyrosine free diet, TD-09362, Harlan Teklad, Custom Research Diets) and water containing sufficient amount of tyrosine (0.45g/L) except for the periods of experimentation. All animals were allowed to acclimatise to the non-SPF animal unit for at least 7 days prior to experimentation. On the day of testing, mice were removed from non-SPF animal unit and accommodated in the behavioural testing room for 1-2 h prior to testing. Lighting was controlled on a 12 L : 12 D cycle (light on: 07.30 h, lights off: 19.30 h; temperature was maintained at $20\pm 2^{\circ}\text{C}$, and relative humidity at 50-80%). All experimental testing was carried out between 10.00am and 16.00pm.

2.2.7.2.2 Measuring home cage activity

The measurement of home cage activity was carried out with the use of automated home-cage activity monitors. Mice of each genotype were placed individually in a standard plastic-bottomed cage that was placed in a home cage activity monitor. Behaviour was recorded automatically with passive infrared sensors mounted above the floor of the test cage. All of the activity in the cage was measured by interruption of a sensor beam. As well as these measurement scales were introduced for quantification of locomotor activity, stereotypy and grooming behaviour. For locomotor activity and stereotypy time sampling method with 5 minute interval recordings for the duration of one hour was adopted. For grooming behaviour a continuous recording of behaviour was carried out for the duration of one hour. Rating scales employed to score the stereotyped behaviour, locomotor activity and grooming are given in Table 2.14, Table 2.15 and Table 2.16 respectively. Sniffing was defined as rhythmic movement of the snout and head along the cage wall or floor, accompanied by rapid movement of vibrissae. Rearing was defined as when mouse raised both front paws from the floor of the test area. Grooming observed was both

continuous and discontinuous interrupted by locomotion and exploratory activity. It consisted of either just head and snout grooming or initial head and snout grooming with the front paws followed by burrowing of the snout into the body. Chewing is defined as jaw movement not directed at any stimulus. Gnawing was defined as gripping of the wire of the cage between teeth. Licking was defined as tongue protrusion against the cage floor or wall.

Table 2.14 Stereotypy scale

Score given	Behaviour observed
0	Inactive/asleep
1	Awake/alert
2	Locomotion with some sniff/rear/groom
3	Continuous sniff over wide area
4	Continuous sniff in one location
5	Chewing/biting/gnawing/licking/repetitive head movements

Table 2.15 Locomotor Scale

Score given	Behaviour observed
0	Inactive/asleep
1	Awake/alert/stationary
2	Locomotion with some sniff/rear/groom
3	Rapid and continuous locomotion
4	Rapid locomotion with leaping, jumping, climbing or rapid darting around the perimeter of the test cage

Table 2.16 Grooming

Score given	Behaviour observed
0	Inactive/asleep
1	Facial or any other body part groom for a short period of time
2	Facial and any other body part groom for a long period of time

2.2.7.2.2 Measuring open cage activity

Open cage (exploratory) activity was measured by use of Ethovision 3.1 recording and analysis software. Ethovision is an automated video tracking, motion analysis and behaviour recognition system. Mice of each genotype were individually placed into circular arena (71cm in diameter) and exploration and movement of mice were recorded by video camera mounted directly above arena. This video taken was then analyzed by EthoVision software 3.1 to provide numerical data which could be plotted into graphs.

2.2.7.3 Metabolic profiling by NMR spectroscopy of plasma and tissue extracts

2.2.7.3.1 Preparation of plasma samples for NMR analysis

Blood samples (approx. 150µl each) were collected from tail ventral caudal artery of mice (wild-type, heterozygous and homozygous) into eppendorfs containing K₂EDTA (36mg per L of blood) prepared in D₂O. Plasma was separated by centrifugation at 2,000 x g for 10 minutes at room temperature. Each plasma sample (30µl) was then placed into separate eppendorfs, dried by speedy vac and stored at -80°C until analysis.

Before analysis plasma samples were thawed and mixed with 600 µl of D₂O and 5µl of TSP (1mg/ml) solution.

2.2.7.3.2 Preparation of tissue samples for NMR analysis

Mice (wild-type, heterozygous and homozygous for TGT genetrap insertion) were sacrificed by cervical dislocation and brain and liver were removed and separated into 100mg parts which were then rapidly frozen in liquid nitrogen to immediately stop any enzymatic or chemical reactions. These samples were then stored at -80°C until analysis.

For extraction of polar metabolites from tissue acetonitrile method was used. The solvents were prepared ice-cold on the day. The intact frozen tissue was weighed and then homogenized by electric homogenizer in 50% acetonitrile/50% D₂O (v/v) (5ml.g⁻¹ tissue). The tissue sample was then centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was collected and was frozen in dry ice or liquid nitrogen for 30-60minutes. After this period of time it was free dried overnight in freeze drier. In the morning samples were placed back into dry ice and stored at -80°C until analysis. On the day of analysis samples were reconstituted in 650µl of D₂O and 5µl of TSP (1mg/ml) solution. The pellet was kept on ice for lipid extraction.

The lipid extraction by using 75% CDCl₃ / 25% CD₃OD (1ml) was carried out by using pellet that remained from precious aqueous extraction. The precipitated protein was span down at 12,000 x g for approximately 10 minutes. The solvent was then removed by the use of speedy vac and sample was stored at -80°C until analysis. On the day of analysis the lipid soluble extract was reconstituted in 650µl of deuterated chloroform/methanol (75%/25%) and 5µl of TSP (1mg/ml).

2.2.7.3.3 Processing of tissue and plasma extract for NMR

For processing of the samples for tissue and plasma by NMR collaboration was set up with Dr. John O'Brien (Chemistry Department), Dr. Kenneth Hun Mok (Biochemistry Department) and Dr. Jung Mo Young (Biochemistry Department).

On the day of analysis samples were prepared and pipetted into 5mm NMR tubes. These were then analysed by Dr. John O'Brien on Bruker Avance II 600 Hz NMR with a TCI cryoprobe which has an inverse triple resonance (^1H , ^{13}C and ^{14}N) channel and separate ^{13}C channel. After processing Dr. Kenneth Hun Mok's group aided in analysis of data.

Chapter 3

Queuine deficiency affects tyrosine production in human HepG2 cells

3.1 Introduction

Experiments carried out by Marks and Farkas [64] in 1997 on germ-free mice demonstrated that queuine is an essential micronutrient in the absence of a dietary source of tyrosine. Mice placed on a tyrosine and queuine free diet displayed a number of behavioural symptoms such as squinting, convulsion and eventually death. This effect was reversible by the introduction of either tyrosine or queuine back into the diet; the former result suggesting that the uptake and utilisation of tyrosine is not influenced by queuine status and, by extension, that tyrosine biosynthesis from phenylalanine is the most likely cause for the defect. To this day the relationship between queuine and tyrosine is not understood.

In eukaryotic organisms tyrosine is produced from phenylalanine in a hydroxylation reaction carried out by the PAH enzyme and the reaction requires the natural cofactor BH_4 . In the process of this hydroxylation reaction BH_4 is oxidized to $\text{BH}_4\text{-}4\alpha$ -carbinolamine and an oxygen molecule is transferred to phenylalanine resulting in a formation of tyrosine. Tyrosine can be further hydrolysed by the TH enzyme resulting in the biosynthesis of catecholamines; norepinephrine, epinephrine and dopamine.

Various possibilities can be proposed to explain the link between queuine and tyrosine production. Queuine might have some effect on the PAH protein, whether by way of aiding the activity or function of the enzyme. Alternatively, the removal of queuine from germ-free mice placed on a tyrosine-free diet could cause a severe demand for tyrosine production which causes an increased turnover of PAH protein with the concomitant result of decreased tyrosine production to a fatally low level. A further point for consideration is that it may not be queuine but queuosine-tRNA that has an effect on PAH.

Interestingly, both queuine and BH_4 are synthesized from GTP, and therefore share some degree of structural similarity. This in turn suggests that queuine might have an ability to bind to the active site of PAH and promote the adoption of a more stable

configuration; a possibility we investigated by carrying out kinetic studies on recombinant PAH protein. Queuine could also conceivably affect BH₄ biosynthesis or regeneration thereby affecting tyrosine production, and the production of catecholamines and the indoleamine neurotransmitter serotonin (Figure 3.1).

Work carried out by Ilana Berstein in our laboratory showed that queuine has no effect on PAH transcription or translation in human HepG2 and rat H4IIC hepatoma cells, as determined by quantitative RTPCR and Western blotting. To study the effect of queuine on tyrosine production *in vivo*, queuine sufficient and deficient HepG2 cell lines were created by altering the growth medium conditions. Importantly, HepG2 cells have been previously shown to contain BH₄ and the enzymes associated with its synthesis and recycling [139-147].

Before undertaking this study a literature review was carried out to ascertain the queuine levels found in growth medium. Mediums supplemented with 10% foetal bovine serum (FBS) contain 1 to 2 x 10⁻⁸ M of queuine [51], which in cultured cells is sufficient to allow for full modification of tRNA [148-149]. On the other hand, Horse Serum (HS) is essentially queuine free [150] and so HepG2 cells grown in HS would become deficient in queuine. It was felt that FBS and HS differed significantly, in both known and unknown ways, making them unsuitable for comparative purposes. Therefore, another means to alter queuine levels was sought by growing HepG2 cells in UltraCULTURE™ Serum-free Medium with or without the addition of chemically synthesized queuine. Ultraculture Medium (UC) is considered to be a complete all purpose serum-free medium which can support the growth of a wide variety of both non-adherent and adherent cell lines. It was also necessary to ensure that the laboratory stock of queuine (a gift from Dr. Susumu Nishimura, Tsukuba University, Japan) was still intact as it was synthesized almost 30 years ago.

To measure tyrosine production *in vivo*, HepG2 cells were incubated with radiolabelled phenylalanine and the total cytosolic extracts were analysed by HPLC. Radiolabelled phenylalanine and tyrosine can be separated by reversed phase chromatography on a C18 column due to their differing hydrophobic characteristics. Tyrosine is slightly more hydrophilic and is eluted first. The quantification of radiolabelled tyrosine was made possible by analysing the radioactivity counts of fractions eluted from the C18 column by a scintillation counter. Attempts were also made

to measure BH₄ levels by HPLC in HepG2 cells grown under queuine deficient and sufficient conditions.

3.2 Results

3.2.1 Assessment of queuine levels in HepG2 cells

The method of choice for measuring queuosine levels in tRNA is Liquid chromatography-mass spectrometry, a technique that involves physical separation by HPLC coupled to a mass spectrometer. However, this method requires special equipment and expertise that was not available to us. Therefore, in the course of these studies an improved enzymatic method was devised to measure queuosine tRNA, based on the earlier method described by Nishimura and colleagues [49-50, 151].

This method is based on the ability of the *E.coli* TGT enzyme to insert radiolabelled guanine into tRNA that is not modified by queuosine. Therefore, a low level of guanine incorporation means that there is a high level of queuosine modification. The tRNA was extracted from each of queuine deficient or sufficient HepG2 cell lines and was charged with radioactive guanine. In order to separate the charged guanine-tRNA from free radiolabelled guanine the sample was deproteinated and passed through a small home-made DE52 spin column. The tRNA bound to DE52 resin and free guanine was washed away. The charged tRNA was then eluted and easily quantified by scintillation counting.

To ensure that this method could correctly evaluate queuosine levels in tRNA a control experiment was set up. Different concentrations of yeast tRNA were charged with ^{14}C -guanine and separated by passing through DE52 spin columns (1mL) (Figure 3.1A). The concentration of yeast tRNA loaded on the DE52 spin column (1mL) had a linear relationship with respect to the amount of guanine incorporated signifying that the amount of TGT enzyme used was sufficient to modify up to 2 absorbance units of tRNA and that the DE52 column has adequate capacity to retain this quantity of tRNA.

To examine the influence of growth medium on the queuosine-tRNA level and the status of our laboratories stock of queuine, HepG2 cells were grown for three passages in UC medium, UC medium supplemented with 300 nM queuine, DMEM supplemented 10% FBS, and DMEM supplemented with 10% HS. Approximately 10 absorbance units (a.u.) of tRNA were purified from each condition and 2 a.u. were used per guanine incorporation assay. This particular amount was chosen since in the control yeast tRNA

experiment it is within the linear range of the assay and it gives an acceptably high number of disintegrations per minute.

The resultant graph (Figure 3.1B) shows high levels of guanine incorporation in tRNA extracted from HepG2 cells grown in UC and HS mediums and low levels of guanine incorporation in tRNA extracted from HepG2 cells grown in UC+Q and FBS mediums (See Figure 3.1 B for Mean values, SD and SEM). These results indicate that UC is a suitable growth medium to generate queuine and queuosine-tRNA deficient cells and that the stock of queuine in the laboratory is chemically intact.

3.2.2 Assessment of queuine levels in HepG2 cells by LCMS

In order to validate the enzymatic method to measure queuosine modification of tRNA a collaboration was created with Vimbai Chikwana (Prof. Dirk Iwata Reuyl's group in Portland State University) to analyse tRNA extracted from HepG2 cells grown under different conditions by LCMS. HepG2 cells were grown for three passages in UC medium, UC medium supplemented with 300 nM queuine, DMEM supplemented 10% FBS, and DMEM supplemented with 10% HS. Approximately 10 absorbance units (a.u.) of tRNA were purified from each condition and freeze dried and sent to Vimbai Chikwana for analysis.

The control LCMS mass spectra of tRNA extracted from *E.coli* shows the presence of queuosine and preQ1 as well as an ion at 295 m/z, which can usually be found when either queuosine or preQ1 are present (Figure 3.2 A). The samples of tRNA were first run through an HPLC and then analysed by mass spectrometry (Figure 3.2). Since FBS is known to contain queuine it is a good control for the presence of queuosine modified tRNA. The results here show that tRNA from HepG2 cells grown in FBS medium does indeed contain the queuosine modification (Figure 3.2 B) confirming our results. The tRNA extracted from HepG2 cells grown in UC+Q, was shown to contain queuosine modified tRNA (Figure 3.2 C) at a level that was comparable to FBS if not better.

The tRNA extracted from HepG2 cells grown in UC and HS was shown to contain no queuosine modified tRNA (Figure 3.2 D and E). The signal at 410 m/z is the same as the background and the ion at 295 m/z was also absent from these two samples. Therefore, as our previous enzymatical analysis showed, UC medium and HS do not contain queuine.

3.2.3 The effect of queuine on tyrosine production *in vivo*

Previously, studies carried out by Ilana Berstein demonstrated that queuine does not influence the transcription or translation of the PAH protein. The tyrosine biosynthesis pathway is dependent on the BH₄ cofactor which acts as a reducing agent for the PAH protein. Together with Ilana Berstein, an investigation into the effect of queuine on tyrosine biosynthesis *in vivo* was carried out. The experiment was repeated by me with the use of protocols devised by Ilana Berstein. HepG2 cells were grown to 80% confluence in UC medium with or without queuine and in DMEM medium supplemented with 10% FBS (Figure 3.3). One hour before the addition of radiolabelled phenylalanine, cells were depleted of phenylalanine and tyrosine by incubation in customised UC medium that contains no tyrosine or phenylalanine. Cells were then incubated with increasing concentrations of ¹⁴C-phenylalanine to allow cells to take it up from the medium. Total cell extracts were recovered and the intracellular tyrosine and phenylalanine were analysed by reversed phase separation on a C18 column by HPLC (Figure 3.4A.). This separation is possible due to the difference of hydrophobic character of amino acids; tyrosine being slightly more hydrophilic and therefore eluting from the column first. The eluted fractions were collected and the amount of radiolabelled tyrosine and phenylalanine were separately measured with the use of a scintillation counter. The results obtained show that the production of tyrosine after 30 minute incubation with phenylalanine is reduced in HepG2 cells grown in UC medium when compared to that of HepG2 cells grown in UC medium with queuine and FBS medium (Figure 3.4 B (See Table 3.1 for Mean values, SD and SEM)). The uptake of phenylalanine was also examined and it was found to be linear with respect to the phenylalanine concentration supplied to the medium (Figure 3.4 C, (See Table 3.1 for Mean values, SD and SEM)).

The decrease in tyrosine biosynthesis over time suggests that queuine might have an effect on the activity of the phenylalanine hydroxylase enzyme or on the BH₄ biosynthesis or regeneration pathways. This assay was performed in triplicate for each concentration and the experiment was repeated 5 times with the same result.

3.2.4 The effect of queuine and queuosine tRNA on the enzymatic actions of recombinant phenylalanine hydroxylase

To obtain sufficient amounts of high purity PAH protein, recombinant enzyme was expressed and purified from *E. coli*. Firstly, an overnight *E. coli* culture (5ml) was grown up and transferred to 500ml of LB medium. This was grown until the absorbance at 600nm reached a value of between 0.6 and 0.8 a.u. Protein production was initiated by the addition of IPTG and incubation at 30°C for 4 hours. The pellet was collected, lysed and cell debris removed by centrifugation. The PAH protein was then purified from supernatant using affinity chromatography on amylose resin. The protein was purified with an MBP-tag and at different stages of the PAH purification, fractions were collected for analysis by SDS-PAGE. The protein fractions were stored at -80°C and the concentrations were determined as described in section 2.2.4.1. Approximately 10 mg of protein was obtained from 4 litres of cell culture. Total protein in each of these fractions was separated on SDS-PAGE and visualised by coomassie blue staining (Figure 3.5 A).

The purified protein was then used to investigate the effect of queuine and queuosine tRNA on the enzymatic actions of recombinant PAH protein. Queuine is derived from a GTP molecule as is BH₄ [152] so there is a possibility that queuine or queuosine tRNA have an ability to bind the active site of PAH inducing a stabilised form. Kinetic studies were carried out with and without queuine, with tRNA on its own or tRNA containing modified queuosine base (Figure 3.5 B). The PAH activity assay was based on the measurement of tyrosine production from phenylalanine. Tyrosine production can be measured by obtaining initial rates of the reaction at 275nm on a UV-Vis spectrophotometer. Studies were performed with three different concentrations of chemically synthesized queuine and with tRNA on its own or tRNA modified with queuosine. Increasing concentrations of 6(R)-BH₄ were used and the initial rates of the reaction were recorded using a chart recorder attached to the spectrophotometer. Phenylalanine concentration was kept constant at 50 times the value of K_m at 25mM to remove any variability with the second substrate. The initial rates of the reaction were calculated from traces obtained from chart recorder and the results were fitted to the Michaelis Menten equation to graph and calculate the apparent K_m and V_{max} values

(Figure 3.5 C). From the Michaelis-Menten curve fits it is evident that neither queuine or tRNA or queuosine-tRNA had any effect on the activity of the PAH protein.

The tyrosine production activity of PAH protein was also examined in extracts of cells that were grown under queuine sufficient and deficient conditions. This work was done by Ilana Berstein to determine whether queuine has some other contributions such as influencing post-translational modification of the PAH protein. In this regard, PAH protein is known to be a target for post-translational phosphorylation [153] which might suggest that phosphorylation is needed for PAH activation. Also, previous reports in the literature suggested that queuine could enhance a number of growth pathways in the cell, leading to the phosphorylation of transcription factors such as c-fos and myc [57].

In this assay queuine deficient and queuine-modified cell lines were created and passaged onto a 6 well dish. They were cultured in UC medium with and without queuine (final concentration 200nM) and in DMEM with 10% FBS as a positive control. Under these conditions the effect of queuine on PAH activity both as a free base, as well as queuosine modified base in tRNA could be examined. Protein extracted from the cells was incubated with radiolabelled ^{14}C phenylalanine and tyrosine production was examined by thin-layer chromatography (TLC). TLC is a technique that allows the separation of peptides based on their polarity. Highly polar compounds do not migrate as fast within the stationary phase and since tyrosine is a more polar molecule than phenylalanine it is possible to separate these two amino acids by this technique. It was possible to detect tyrosine produced from radiolabelled phenylalanine by the use of autoradiography. The results of this experiment showed that the activity of PAH enzyme remained unchanged in queuine deficient and sufficient conditions (Figure 3.5 D). Therefore, queuine as a free base or as modified base has no effect on PAH protein activity.

3.2.5 The effect of queuine on tetrahydrobiopterin levels in HepG2 cells

The results from the study examining the effect of queuine on tyrosine production *in vivo* suggested that queuine deficiency must affect tyrosine production by another means most probably through influencing BH_4 biosynthesis or regeneration. So it was decided to look at the levels of BH_4 in these cells (Figure 3.6). Unfortunately, each determination

required 10 x T175 flasks or approximately 6×10^8 cells per experiment. HepG2 cells were grown to 80% confluence in UC and UC+Q mediums. The intracellular content was extracted by freeze-thaw action and the levels of BH₄ were measured after iodine oxidation in acidic or alkaline conditions. Total biopterin represents the sum of BH₄, BH₂ and fully oxidized biopterin. Differential oxidation with iodine enables measurement of both total biopterin and BH₄. Under acidic conditions BH₄ and BH₂ are oxidized to biopterin, while under basic conditions only BH₂ is oxidized to biopterin. Thus the difference in biopterin content between the two oxidation steps represents the actual BH₄ levels. The biopterin analysis was carried out by HPLC, where pterins were separated on a Spherisorb ODS-1 column with 5% methanol as the mobile phase. They were detected by their native fluorescence with the excitation wavelength of 350nm and the emission wavelength of 450nm.

Unfortunately, even though this experiment was carried out a number of times the results were always variable and the level of biopterin proved to be too low to be accurately quantified. From the traces shown in Figure 3.6 it can be seen that the biopterin levels in HepG2 cells grown in queuine deficient conditions is higher than that found in HepG2 cells grown in queuine sufficient conditions however, this result was highly variable and the effect of queuine on BH₄ in HepG2 cells could not be determined with certainty.

3.2.6 The effect of TGT inhibition and on tyrosine production

A known inhibitor of the TGT protein, which is also capable of stopping queuosine formation in vivo, is 7-methylguanine [154-155]. In order to distinguish the effect of free queuine base from queuosine modified tRNA on tyrosine production, 7-methylguanine was used to treat HepG2 cells (Figure 3.7, (See Figure 3.7 B for Mean values, SD and SEM)). Interestingly, addition of 7-methylguanine did show a reduction in tyrosine production (385.77 ± 3.74 fmol per mg total protein) when compared to the queuine sufficient control (458.15 ± 10.15 fmol per mg total protein) (See Table 1). Presuming that 7-methylguanine inhibited TGT activity this result would indicate that queuosine-tRNA is the component that is required for proper tyrosine production. It is possible however that inhibition of TGT activity could be due to direct binding of 7-

methylguanine to TGT active site or due to competitive binding with queuine to another allosteric site on TGT protein or even on some other enzyme.

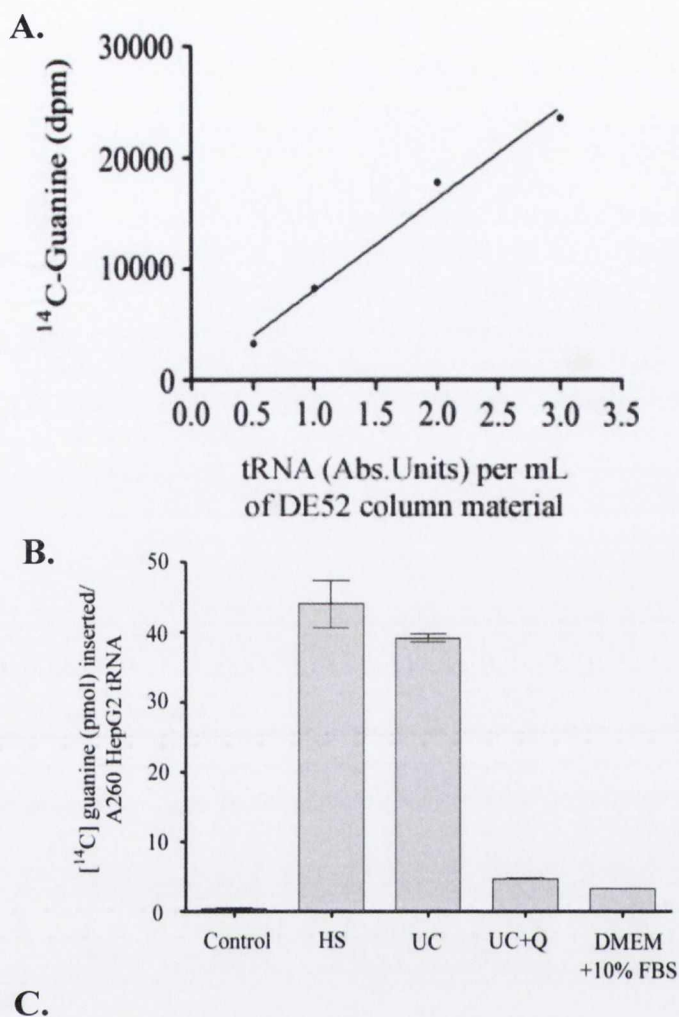


Figure 3.1 The effect of growth medium on Q-tRNA levels in HepG2 cells.

(A) Standard Curve for ^{14}C -Guanine incorporation into yeast tRNA. Increasing concentrations of yeast tRNA were charged with $72.73\mu\text{M}$ ^{14}C -Guanine by using $40\mu\text{g/ml}$ *E.coli* TGT enzyme. **(B)** tRNA queuosine levels in HepG2 cells grown in various culture mediums. tRNA was extracted and 2 Abs units charged with ^{14}C -guanine by *E.coli* TGT as described in A). HS, DMEM with 10% horse serum; UC, Ultraculture™ serum free medium; UC+Q, Ultraculture medium containing 200 nM queuine; DMEM +10% FBS, DMEM medium with 10% fetal bovine serum. **(C)** Statistical analysis of the TGT activity assay in HepG2 cells (Figure 3.1 B.)

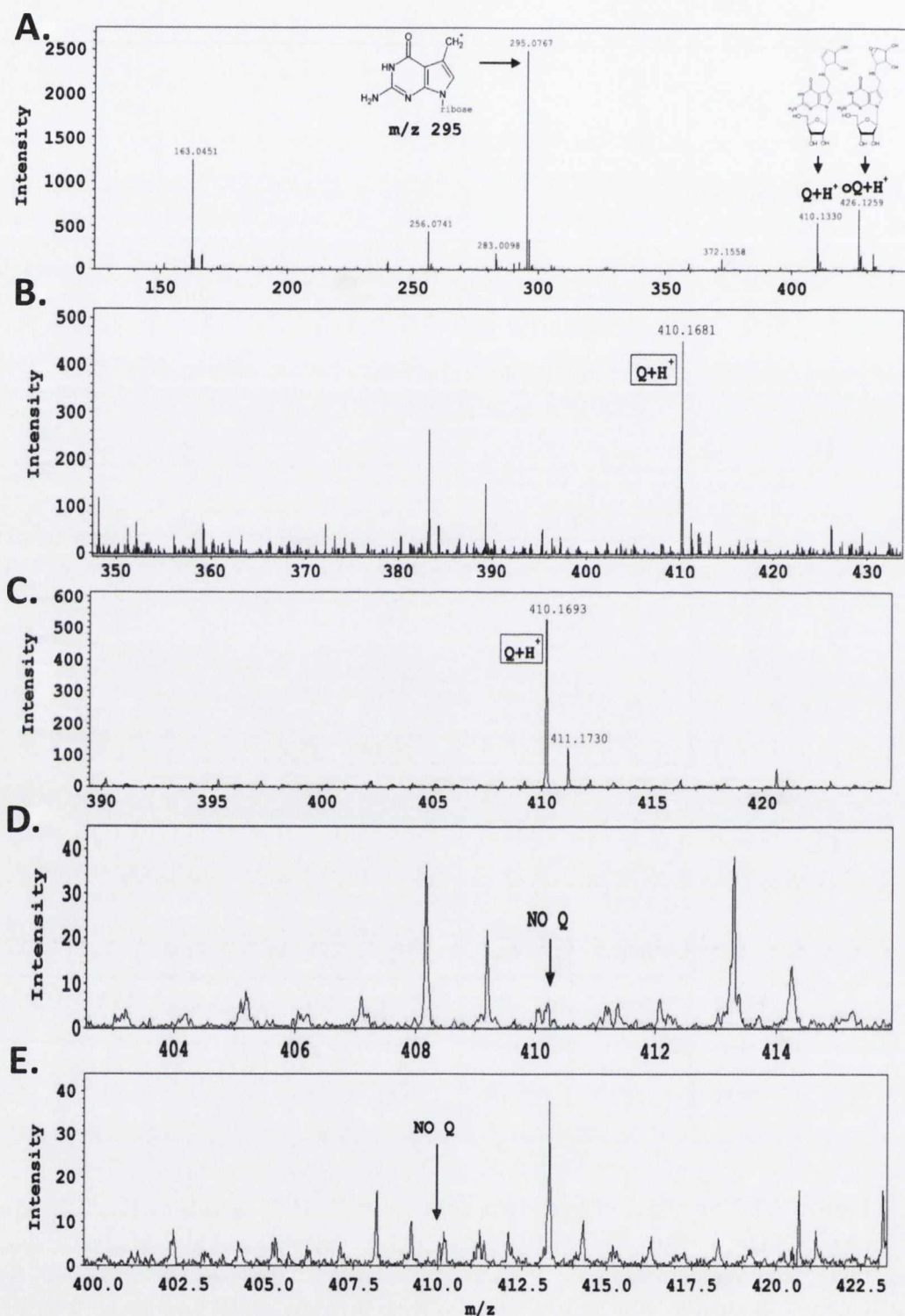


Figure 3.2 LCMS quantitation of Q-tRNA levels in HepG2 cells grown in different mediums. LCMS mass spectra of tRNA isolated from (A) *E. coli* showing the presence of Q at 410 m/z and the Q-precursor preQ₁ at 426 m/z (oQ); (B) HepG2 cells grown in FBS; (C) HepG2 cells grown in ultraculture medium supplemented with queuine (UC+Q); (D) HepG2 cells grown in ultraculture medium (UC); (E) HepG2 cells grown in DMEM with 10% horse serum. LCMS analysis was kindly carried out by Vimbai M. Chikwana, Department of Chemistry, Portland State University

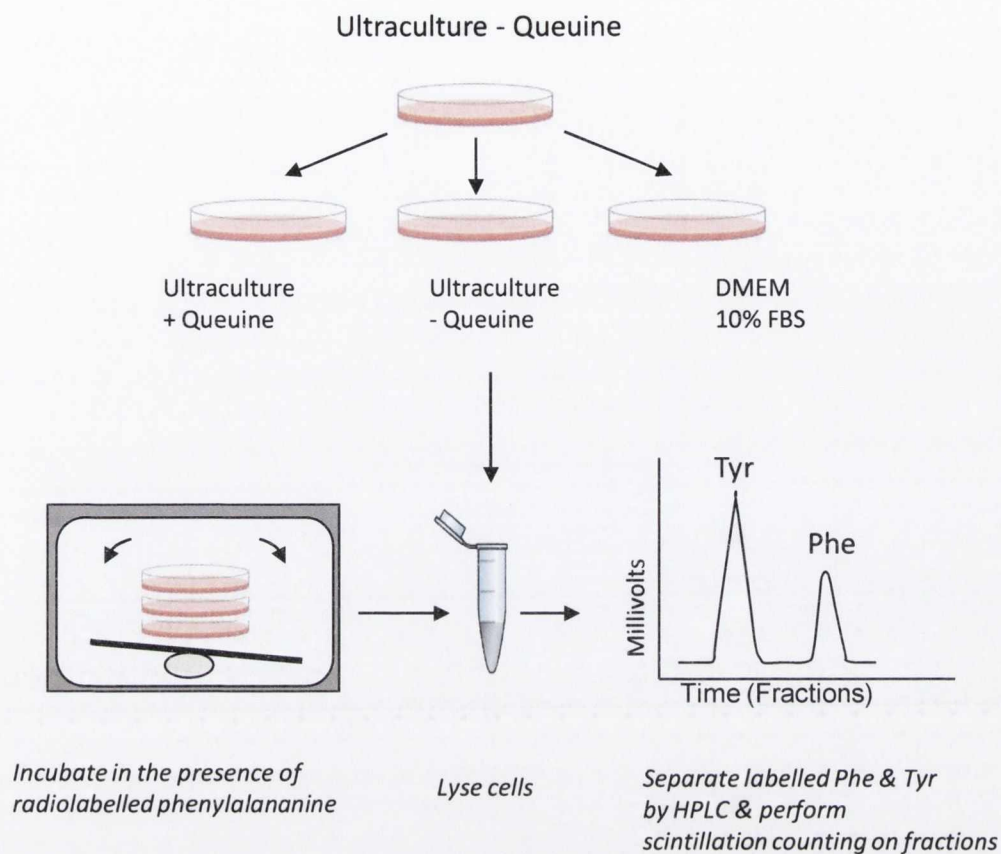


Figure 3.3 Method to examine the influence of queuine on tyrosine production in HepG2 cells. HepG2 cells were cultivated to 80% confluency in queuine deficient medium (Ultraculture minus queuine), queuine sufficient medium (Ultraculture plus queuine) and DMEM with 10% FBS. Cells were then incubated in the presence of ^{14}C -phenylalanine with constant shaking and were lysed with 10% TCA. The radiolabelled tyrosine and phenylalanine were separated on a Zorbax 300SB-C18 column by HPLC. Fractions of 1ml were collected and the levels of radiolabelled phenylalanine and tyrosine detected by ionizing radiation using a scintillation counter.

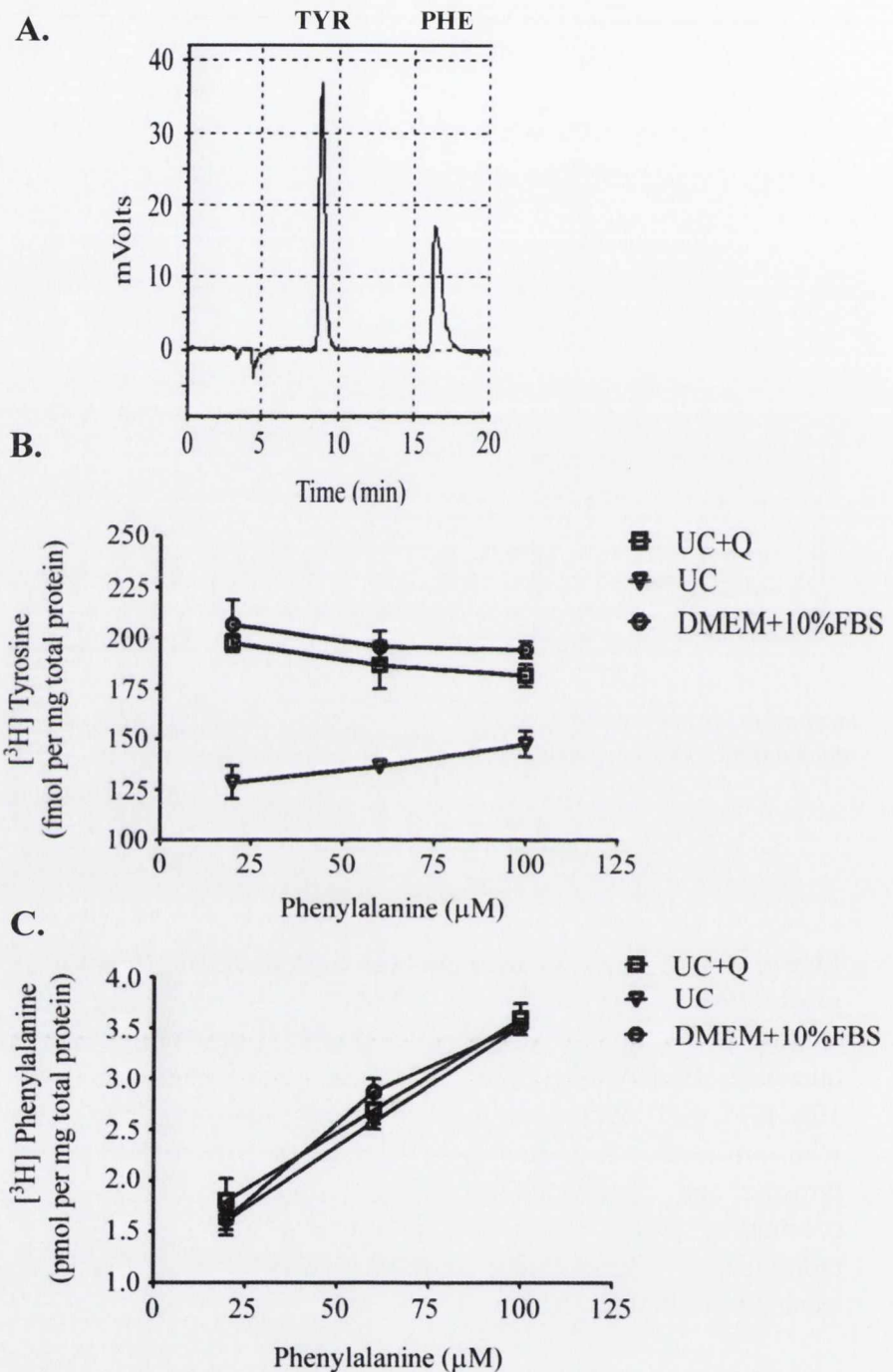


Figure 3.4 Queuine deficiency leads to a decrease in tyrosine production in HepG2 cells. (A) Representative HPLC elution profile of tyrosine (first) and phenylalanine (second) from a C18 column. HepG2 cells that had been grown in Ultraculture medium in the absence (UC) and presence of queuine (UC+Q) or in DMEM containing 10% FBS (DMEM + 10% FBS) were incubated with increasing concentration of radiolabelled phenylalanine. Intracellular tyrosine production (B) and phenylalanine concentrations (C) were measured by collection of HPLC fractions followed by scintillation counting. *This work was performed with the use of protocols established by Ilana Berstein (See Table 3.1 for Mean values $\pm$ SEM)*

A.

Phenylalanine (μ M)	[³ H] Tyrosine (fmol per mg total protein)					
	UC+Q		UC		DMEM+10% FBS	
	<i>Mean</i>	<i>SEM</i>	<i>Mean</i>	<i>SEM</i>	<i>Mean</i>	<i>SEM</i>
20	197.41	4.34	128.80	8.23	206.67	11.99
60	186.53	11.68	136.79	1.00	195.88	7.43
100	181.59	5.57	147.64	6.36	194.23	4.09

B.

Phenylalanine (μ M)	[³ H] Phenylalanine (pmol per mg total protein)					
	UC+Q		UC		DMEM+10% FBS	
	<i>Mean</i>	<i>SEM</i>	<i>Mean</i>	<i>SEM</i>	<i>Mean</i>	<i>SEM</i>
20	1.80	0.22	1.61	0.09	1.627	0.16
60	2.70	0.14	2.58	0.07	2.88	0.12
100	3.59	0.15	3.52	0.05	3.53	0.04

Table 3.1. Data obtained from *in vivo* assay of intracellular tyrosine levels in HepG2 cells. (A) Statistical analysis of intracellular tyrosine levels (Figure 3.4 B.) and (B) phenylalanine concentrations (Figure 3.4 C) measure by HPLC separation, HPLC fraction collection and scintillation counting.

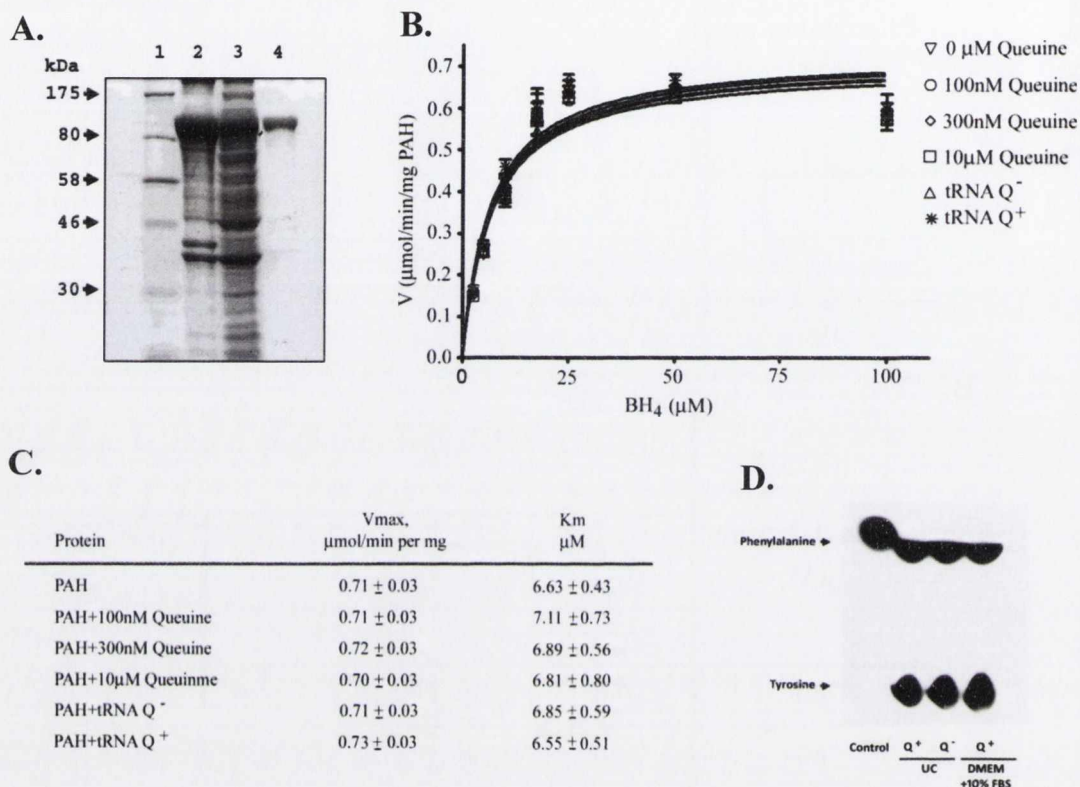


Figure 3.5 Queuine and Q-tRNA have no influence on the enzymatic activity of the phenylalanine hydroxylase enzyme. (A) SDS/PAGE analysis of recombinant phenylalanine hydroxylase protein overexpressed in BL21-RIL cells. Lane 1: protein markers; lane 2: insoluble protein from the sonicated *E. coli* pellet; lane 3: soluble protein from cell lysate supernatant; lane 4: purified PAH protein eluted from MBP column. (B) Kinetic analysis of the effect of various concentrations of queuine, tRNA and Q-tRNA on the recombinant PAH protein. The initial rate of reaction of PAH enzyme was measured with increasing concentrations of BH₄. The data points were fitted to a Michaelis-Menten curve using the prism graphics software. The apparent K_m and V_{max} were calculated (C) from the graphs using the computer program GraphPad Prism. (D) Effect of queuine on the activity of PAH protein *in vitro* (the results for panel D were produced by Ilana Bernstein). HepG2 cells were cultured in Ultraculture medium in the absence (Q-) and presence of queuine (Q+), and in DMEM medium with 10% FBS as a positive control. Cytosolic protein was isolated (100μg) and PAH activity determined through the conversion of ¹⁴C phenylalanine to ¹⁴C tyrosine. The results were visualised by separation of the amino-acids on thin-layer chromatography followed by autoradiography.

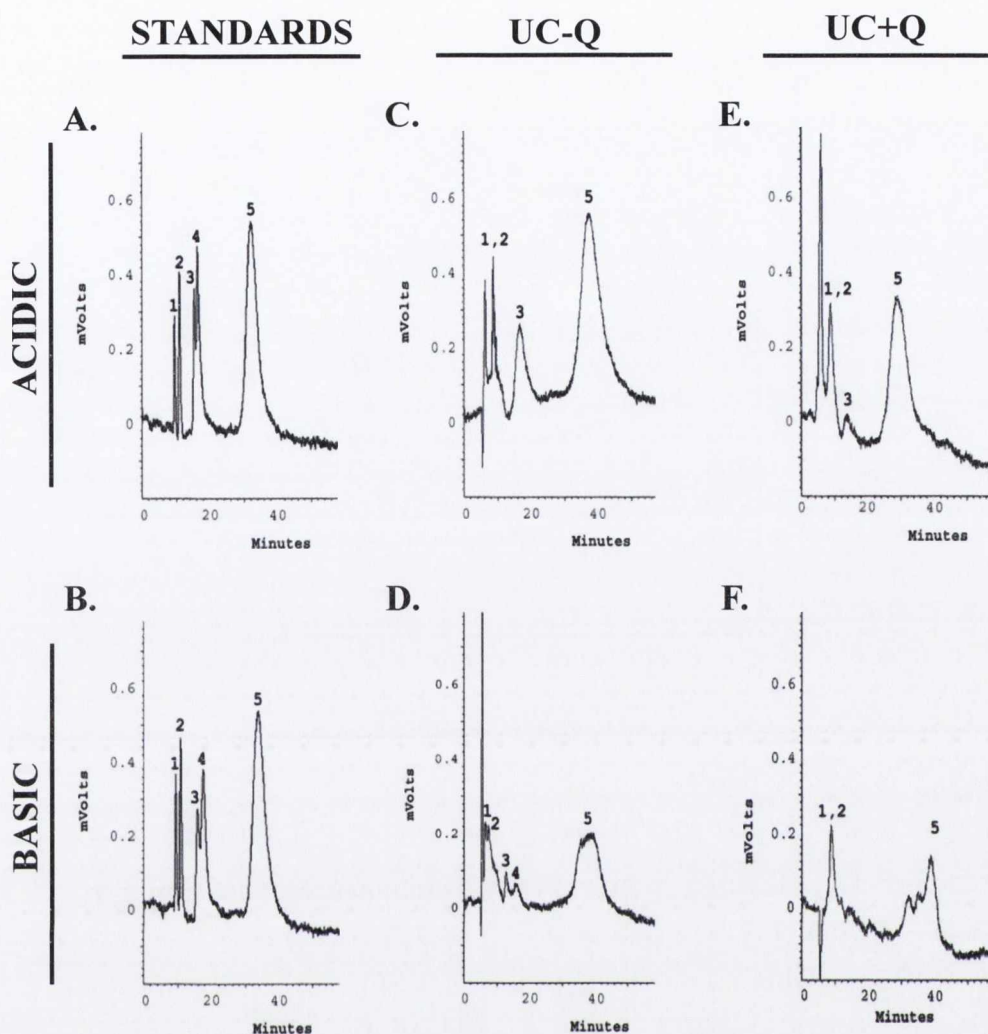
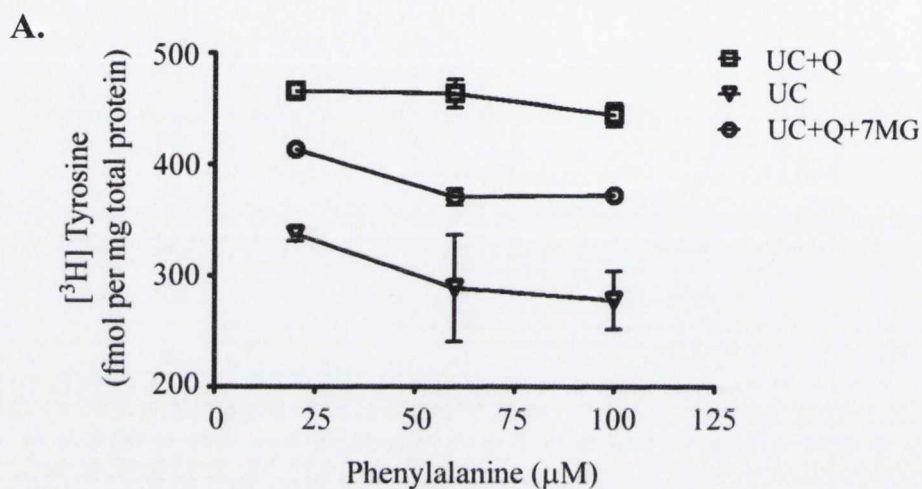


Figure 3.6 Examining the effect of queueine on tetrahydrobiopterin levels in HepG2 cells. HPLC chromatographs of a standard mixture containing 7-neopterin (1), neopterin (2), 7-biopterin (3), biopterin (4) and pterin (5) following iodine oxidation under acid (A) or alkali (B) conditions. Reversed-phase separation was carried out on a Spherisorb ODS-1 column eluted with 5% methanol - 95% water at 0.6ml/min. Pterins were detected by their native fluorescence with an excitation wavelength of 350 and an emission wavelength of 450nm. Chromatographs showing pterin separation from HepG2 extracts made from cells grown in Ultraculture medium alone (UC-Q) following acidic (C) or basic (D) iodine oxidation or in UC medium supplemented with 300nM queueine (UC+Q) following acidic (E) or basic (F) iodine oxidation.



B.

Phenylalanine (μM)	[³ H] Tyrosine (fmol per mg total protein)					
	UC+Q		UC		UC+Q+7MG	
	Mean	SEM	Mean	SEM	Mean	SEM
20	465.83	7.40	337.76	6.49	413.91	3.35
60	463.76	12.825	288.96	47.92	370.90	7.33
100	444.85	10.224	278.17	26.09	372.49	0.53

Figure 3.7 Queuosine modification of tRNA is link to phenylalanine to tyrosine conversion. HepG2 cells were cultivated in ultraculture medium that was unsupplemented (UC), supplemented with queuine (UC+Q) or supplemented with both queuine and 7-methylguanine (UC+Q+7MG), a weak inhibitor of the queuine incorporating activity. Note that 7-methylguanine was added to the cells 48 hours prior to, and after the addition of queuine. To examine phenylalanine to tyrosine conversion cells were incubated with increasing concentrations of ¹⁴C phenylalanine for 1 hour, cell extracts were prepared and intracellular tyrosine levels measured by HPLC followed by scintillation counting. *This work was performed with the use of protocols established by Ilana Bernstein.* (B) Statistical analysis of data obtained during measurement of intracellular tyrosine levels in the presence of 7-methylguanine (Figure 3.7 A.).

3.3 Discussion

The purpose of this study was to explain the link between tyrosine and queuine that was initially observed in 1997 by Marks and Farkas [64], where they had shown that queuine is essential when tyrosine is removed from the diet. This original study was carried out in laboratory mice which may have carried an unusual gene mutation or alternatively the effect may be specific to mice. Therefore, to make sure that this result is applicable to humans, attempts were made to reproduce the effect in HepG2 cells, a human hepatoma cell line.

Before carrying out this experiment it was necessary to confirm that the mediums used for cultivating queuine sufficient or deficient HepG2 cell lines do or do not contain queuine. A simple and straight forward method was devised to measure the presence of queuosine modification in tRNA, which showed that tRNA from HepG2 cells grown in UC and HS mediums does not contain the queuosine modification, whereas tRNA from HepG2 cells grown in UC+Q and FBS do. These results were confirmed by LCMS analysis carried out by Vimbai Chikwana, Portland University. It was previously known that FBS contains queuine and HS does not, however it was not known whether or not UC medium does and if the addition of chemically synthesized queuine would be sufficient for a generating fully queuosine modified tRNA. Here the results show that queuosine incorporation into tRNA of HepG2 cells grown in UC+Q occurs to the same extent as tRNA of HepG2 cells grown in FBS.

Once the queuine contents of the mediums had been established it was possible to use HepG2 cell lines to examine the effect of queuine on tyrosine production and phenylalanine uptake. Tyrosine production after 30 minutes was found to be significantly lower in HepG2 cells grown in the absence of queuine relative to HepG2 cells grown in queuine sufficient conditions, while phenylalanine uptake was found to be directly proportional to the amount of phenylalanine added, meaning that there were no changes to the transport mechanisms for this amino acid. There was an approximate 30% decrease in tyrosine production in HepG2 cells grown in UC medium without queuine at the end of 30 minute incubation time. If the corresponding decrease in tyrosine was found in rodents it would be enough to cause symptoms like those found by Marks and Farkas [64] when maintaining germ-free mice on a tyrosine and queuine free diet. The levels of

tyrosine production were found to be constant with the increasing phenylalanine concentrations suggesting that the PAH enzyme in the cells was saturated by the concentrations of phenylalanine given and working to full capacity.

It was then decided to look at the effect of queuine and tRNA with or without the queuosine modification on the catalytic ability of the PAH enzyme. The results obtained showed that queuine in its free base form or as a nucleoside in tRNA does not have any role in modulating the activity of the PAH protein. To make sure that PAH protein is not being affected post-translationally it was decided to carry out an *in vitro* cell assay. HepG2 cells were grown in queuine sufficient and deficient conditions. Total protein cell extracts were prepared and tyrosine production was analysed by TLC. It was shown that the activity of PAH protein remained the same under queuine sufficient and deficient conditions.

Tyrosine production by the PAH protein requires BH_4 . So the next logical step was to look at the effect that queuine has on BH_4 levels. However, even though the separation of pterins was possible, the results proved to be too variable and the levels of biopterin were too low to be quantified accurately. So the possibility still exists that queuine might have an effect somewhere in the BH_4 regeneration or biosynthesis pathways.

Next, in order to distinguish whether queuine has an effect on tyrosine production as a free base or as queuosine incorporated into tRNA, it was decided to add a known inhibitor of TGT enzyme activity, 7-methylguanine. Previously, 7-methylguanine was used as an inhibitor of queuosine modification in cells in culture, but importantly the result published by Elliot et al [156] showed that queuine uptake is not inhibited by 7-methylguanine in cultured human fibroblasts. Interestingly, the results of our study showed 7-methylguanine reduced tyrosine production after 1 hour incubation when compared to queuine sufficient control (see Table 1). This suggested that it is queuosine-tRNA and not free base of queuine that is the component required for proper tyrosine production.

From these studies, together with the studies carried out by Ilana Berstein, it is evident that queuine does not affect the activity, transcription or translation of the PAH protein. However, queuine, and more specifically queuine inserted into tRNA, does affect tyrosine production although the exact cause of the defect in the tyrosine biosynthetic pathway could not be deduced

Chapter 4

Queuosine modified tRNA is required for normal production of tyrosine in mouse

4.1 Introduction

The studies previously carried out by Reyniers *et al.* [39] showed that the complete depletion of queuosine-tRNA from germ-free mice has no adverse effect. So, for the sake of our study, it was decided to create a TGT gene-trap mouse, the work for which was initiated by other people in our group. These mice contain no queuine modified tRNA and allow us to look more closely into the individual physiological functions of the free queuine base and queuine modified tRNA.

In this study the TGT gene-trap mice were characterized and the effect of queuine on tyrosine production investigated. As mentioned before, PKU is a disease characterized by the accumulation of phenylalanine due to a deficit in the PAH protein or defects in either the recycling or biosynthesis of BH₄ [91]. Individuals diagnosed with PKU must adhere to a low phenylalanine diet as patients who have failed to do this—before the disease process was well understood—developed mental retardation, asthma, headache, hypopigmentation growth retardation and other neurological symptoms [76-77] such as hyperactivity, hypoactivity and seizure [75, 79-80].

From our experiments carried out on HepG2 cells it was evident that queuine has a role in tyrosine production so it was decided to look at tyrosine production in mice deficient in queuosine modified tRNA. This allowed us to distinguish which form of queuine, free base or nucleoside, is responsible for the defect in tyrosine production. It was also interesting to see whether this deficit in tyrosine production leads to phenylalanine accumulation and whether this accumulation can be linked to PKU symptoms. The most prominent cause of PKU is a defect in PAH so it was necessary to firstly confirm the result obtained in HepG2 cells using TGT gene-trap animals; that the expression and activity of the PAH enzyme is not altered by queuine status.

Defects in the recycling or biosynthesis of BH₄ can also cause symptoms of HPA [91], as well as several other neurological disorders such as Dopa-responsive dystonia and depletion of monoamine neurotransmitters [101]. A deficit in BH₄ has also been linked to other neurological diseases that do not have HPA such as Parkinson's disease, depression,

autism and Alzheimer's disease. Low level of BH₄ in human epidermis can also lead to several skin diseases such as vitiligo and Hermansky-Pulack syndrome, which are characterized by low PCD/DcOH activities, accumulation of 6- and 7- bipterin and hydrogen peroxide in skin, and inhibition of tyrosine and melanin biosynthesis [101]. Finally, low levels of BH₄ can be found during the onset of diabetes and coronary heart disease.

Analysis of patients with endothelial dysfunction revealed that they have higher levels of plasma BH₂ while having no change in BH₄, reflecting an increased risk of coronary heart disease, [125]. Other studies have also suggested that BH₂ together with sepiapterin potentiate superoxide formation during the uncoupled reaction of nitric oxide synthase (NOS) in patients with BH₄ deficiency disorders [132-134]. Therefore in this study both the levels of BH₄ and other pterin metabolites, including BH₂, were measured in liver, plasma and urine of wild-type and TGT-genetrap mice.

4.2 Results

4.2.1 The characterization of TGT gene-trap mice

The genotyping of TGT-gene trap mice was carried out by PCR analysis (Figure 4.1 A). A small amount of tissue from an ear punch of 21-day old mice was adequate. Specific primer sets were made to amplify the wild-type and gene-targeted alleles and after the isolation of genomic DNA GoTaq polymerase was used to screen the samples as shown in Table 2.8 and 2.9. The PCR amplicons were resolved on a 2% agarose gel at 120 volts.

The physical characteristics of the mice were examined and revealed no apparent abnormalities as evidenced by the photograph (Figure 4.1 B). The body weight was also examined and homozygous mice showed significant difference ($p < 0.01$) when compared to wild-type mice (Figure 4.1 C, (See Table 4.1A for Mean values, SD and SEM)) by statistical t-test analysis ($n=8$). Examination of organ weight in wild-type and TGT genetrapped mice was also carried out and homozygous mice were found to have significantly lower heart weight ($p < 0.05$) and both heterozygous and homozygous TGT genetrapped mice had significantly lower ($p < 0.05$ and $p < 0.001$ respectively) spleen weight (Figures 4.1 D, (See Table 4.1B for Mean values, SD and SEM)). All other organ weights were comparable across the genotypes. These differences in body and organ weight are potentially very interesting and could indicate a role for queueine in growth and development. Therefore, future studies have been planned to include greater animal numbers for analysis.

4.2.2 Tyrosine production ability of TGT gene-trap mice

From the results in HepG2 cells it was evident that queueine has an effect on tyrosine biosynthesis over time. The generation of TGT gene-trap mice allowed us to look at the effect of queueine on tyrosine production in living organisms. Phenylalanine was administered by intraperitoneal injection (1mg/g body weight) without anaesthesia. Two separate experiments were carried out. In the first experiment the level of tyrosine production was measured in mice kept on normal diet and in the second experiment mice were starved overnight. At different time points, blood samples were collected from ventral caudal artery (Figure 4.2.1 A) and the plasma analysed for levels of tyrosine and

phenylalanine The separation of phenylalanine and tyrosine was carried on a Zorbax 300SB-C18 HPLC column by measuring the absorbance values at 206 nm. Standards were used to confirm the elution times of tyrosine and phenylalanine (Figure 4.2.1 B).

First, the ratio of tyrosine concentration over phenylalanine concentration was examined over time for both experimental conditions (Figure 4.2 .1C and D). If the value of the ratio is larger than one, it means there is more tyrosine present in the sample than phenylalanine which means that most of the phenylalanine is being converted to tyrosine. Conversely, if the value of this ratio is below one it signifies the level of phenylalanine is high relative to tyrosine. The resultant graph of tyrosine over phenylalanine showed that after phenylalanine administration tyrosine biosynthesis is severely compromised over 7 hour time period in TGT gene-trap mice when compared to wild-type mice. There was only a small increase in tyrosine produced from phenylalanine in heterozygous and homozygous mice over time while an increase in tyrosine biosynthesis can be easily observed in wild-type mice with the maximum occurring at 4 hours (Figure 4.2.1 C and D). It has to be noted that the tyrosine production from phenylalanine seems to be equal in all three genotypes in the first hour and after 6 hours.

Tyrosine and phenylalanine levels over the time course in both experiments was also plotted (Figure 4.2.2). Both fasted and fed TGT gene-trap animals (heterozygous and homozygous) showed a decrease in tyrosine production over a period of time from approximately 30 minutes to 6 hour time points (Figure 4.2.2 A and B) and an accumulation of phenylalanine over time (Figure 4.2.2 C and D) relative to wild-type animals. However, these changes were more evident in mice kept on a normal diet rather than starved ones. It could be that mice that were starved overnight were depleted of amino acids, especially those that cannot be synthesized *de novo*. Therefore, the tyrosine formed from the injected phenylalanine may be quickly incorporated into new proteins or metabolised to produce required metabolites such as monoamine neurotransmitters.

4.2.3 Liver PAH levels and activity in wild-type and TGT gene-trap mice

Since tyrosine biosynthesis was shown to be impaired over time in TGT genetrapped mice it was necessary to look at the PAH protein levels and activity. If PAH is found to be the

same in wild-type and gene-trap animals, it signifies that queuine must have an effect somewhere else in the biosynthetic pathway.

Western blot analysis was performed on cytosolic liver fractions using PAH specific antisera raised against recombinant PAH protein (*generated by Coilin Boland in our laboratory*). This showed that there was no difference in the level of PAH expression between wild type and TGT gene-trap mice (Figure 4.3 A). The cytosolic fractions were also used to look at the specific activity of PAH protein in all three genotypes. The PAH activity assay was based on the measurement of tyrosine production from phenylalanine at 100 μ M BH₄ concentration (Figure 4.3 B, (See Figure 4.3C for Mean values, SD and SEM)). This can be measured by recording the rates of reaction at 275 nm on a chart recorder. The 100 μ M concentration of 6(R)-BH₄ was used and phenylalanine concentration was 50 times the value of K_m at 25mM to remove any variability with the second substrate. . From resulting graph (Figure 4.3 B) it is clear that there is absolutely no difference in the activity of PAH enzyme in all three genotypes and queuine..

The possibility also exists that the absence of queuosine modified tRNA is in fact leading to an increase in some inhibitory metabolite rather than directly affecting PAH activity. For example, it has been shown that accumulation of 7-biopterin, a spontaneous product of pterin-4 α -carbinolamine, can inhibit tyrosine formation by PAH [157]. To rule out this possibility, tyrosine production by liver cytosolic extracts (that had been only marginally diluted (Dilution of 1:20/19)) was performed over an hour incubation time and amino acids examined by thin layer chromatography (Figure 4.3 D). Since these samples were not diluted by homogenisation buffer, inhibitory metabolites would be expected to remain in the *in vitro* reaction. It should be noted that an exogenous source of BH₄ was supplied. The results showed that tyrosine production remained unchanged between wild-type and gene-trap mice. It can therefore be concluded that queuosine-tRNA has no effect on PAH protein activity and no inhibitory metabolites accumulate in liver in its absence.

4.2.4 Tetrahydrobiopterin levels in wild-type and TGT gene-trap mice

Since the levels of pterins in HepG2 cells were found to be too low for accurate analysis and tyrosine biosynthesis was found to be compromised over time in TGT gene-trap mice it was decided to measure BH₄ levels in liver cytosolic fractions, plasma and urine from the mice. The biopterin analysis was carried out by HPLC, where pterins were detected

by their native fluorescence upon separation on a Spherisorb ODS-1 column with 5% methanol as a mobile phase.

In contrast to HepG2 cells, the measurements of total biopterin, BH₂ and BH₄ levels were possible and the typical traces obtained for each of the genotypes are shown in Figures 4.5-4.6. Differential oxidation with iodine allowed the measurement of the total level of BH₄. Under acidic conditions BH₄ and BH₂ were oxidized to biopterin, while under basic conditions only BH₂ is oxidized to biopterin. Thus the difference in biopterin content between both oxidation procedures represented the actual BH₄ levels.

Pteridine levels were first measured in cytosolic liver fractions of wild-type, heterozygous and homozygous mice for the TGT genetrap insertion (Figure 4.4, (See Table 4.2 for Mean values, SD and SEM)). There was no significant difference in total liver biopterin or BH₄ levels in all three genotypes (Figures 4.4 G and I, respectively). The only significant difference observed was in the level of BH₂. It was significantly higher ($p < 0.001$) in heterozygous and homozygous TGT genetrap mice when compared to wild-type (Figure 4.4 H).

Next pteridine levels were measured in the urine of wild-type and TGT genetrap mice. From the results obtained it was evident that the levels of total biopterin and BH₂ are significantly higher ($p < 0.001$) in homozygous and heterozygous mice when compared to wild-type (Figures 4.5 G and H, respectively). However there was no significant difference in the level of BH₄ (Figure 4.5 I) (See Table 4.2 for Mean values, SD and SEM).

The level of pteridines was also examined in plasma of wild-type and TGT genetrap mice. This time around there was no significant increase in the total biopterin levels (Figure 4.6 G). However, the levels of BH₂ were significantly increased whereas the BH₄ levels were significantly decreased ($p < 0.001$) (Figures 4.6 H and I, respectively) (See Table 4.2 for Mean values, SD and SEM). Considering these results it is apparent that queuine, and in particular queuosine modified tRNA, plays an important role in maintaining normal pteridine levels in eukaryotes.

4.2.5 The effect of queuine on DHFR activity and TH expression in liver cytosol

In the course of this study it was shown that queuosine-tRNA has an effect on pteridine levels. It was found that the levels of BH₂ are increased in plasma, urine and liver. However, the level of BH₄ was only decreased in plasma. As mentioned before DHFR is an enzyme that has an ability to salvage BH₄ from BH₂. So it was decided to investigate whether the increase of BH₂ in liver is due to the deficiency in DHFR. A spectrophotometric specific activity assay was carried out where NADPH and 7,8-dihydrofolate consumption was monitored at 340 nm with a spectrophotometer. From the graph obtained (Figure 4.7 A, (See Figure 4.7B for Mean values, SD and SEM)) it was evident that there was no difference in the activity of DHFR protein in all three genotypes.

During this study it had also been shown that tyrosine production is compromised over time in TGT genetrapped mice. Tyrosine hydroxylase is an enzyme that is responsible for catalyzing the conversion of tyrosine to DOPA. Therefore it was decided to look at the level of expression of tyrosine hydroxylase in wild-type and TGT genetrapped mice. A western blot analysis was performed on brain fractions using an antisera specific for TH protein (Figure 4.7 C). The results obtained showed no difference in the level of TH expression in all three genotypes.

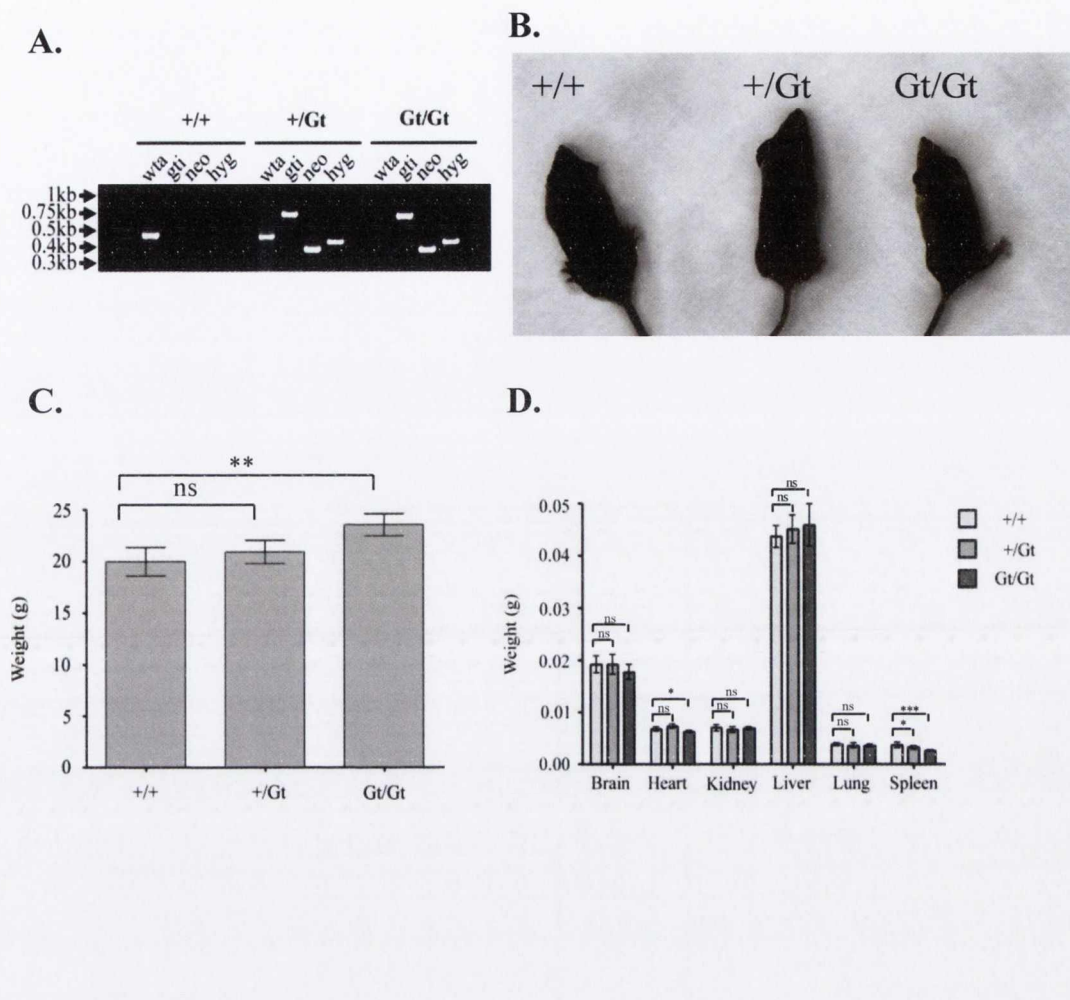


Figure 4.1 *Qtrt1*^{Gt/Gt} mice have normal body and organ weight. (A) Mice were genotyped by PCR analysis of ear punches. By using specific primers made to amplify the wild-type allele and gene-trapped allele it was possible to distinguish wild type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene-trap mice. The PCR amplicons were run on a 2% agarose gel at 120 volts for approximately 40 minutes. (B) A photograph of 21-day old mice of each genotype. (C) Graph depicting the body weight of adult mice of each genotype (n=8) (See Table 4.1A for Mean values, SD and SEM). (D) Graph showing the organ weight of adult mice of each genotype (n=6). *P<0.05, **P<0.01, ***P<0.001, Student's t-test (See Table 4.1B for Mean values, SD and SEM).

A.

	Mean	SD
+/+	19.95	1.38
+/Gt	20.93	1.12
Gt/Gt	23.52	1.07

B.

		+/+	+/Gt	Gt/Gt
Brain	Mean	19.20E-03	19.20E-03	17.76E-03
	SD	3.67E-03	4.33E-03	3.43E-03
	SEM	1.64E-03	1.94E-03	1.40E-03
Heart	Mean	6.79E-03	7.28E-03	6.27E-03
	SD	0.91E-03	0.94E-03	0.50E-03
	SEM	0.41E-03	0.42E-03	0.20E-03
Kidney	Mean	7.00E-03	6.59E-03	6.95E-03
	SD	1.32E-03	1.14E-03	0.67E-03
	SEM	0.59E-03	0.51E-03	0.27E-03
Liver	Mean	43.61E-03	44.98E-03	45.83E-03
	SD	1.87E-05	5.45E-03	10.01E-03
	SEM	7.64E-06	2.22E-03	4.09E-03
Spleen	Mean	3.73E-03	3.18E-03	2.62E-03
	SD	1.87E-05	0.60E-03	0.22E-03
	SEM	7.64E-06	0.24E-03	9.18E-05
Lung	Mean	3.78E-03	3.55E-03	3.5E-03
	SD	1.87E-05	0.98E-03	0.53E-03
	SEM	7.64E-06	0.40E-03	0.22E-03

Table 4.1. Analysis of data obtained during characterization of TGT gene-trap mice (A) Statistical analysis of the body weight (Figure 4.1 C.) and (B) organ weight of adult mice of each genotype (Figure 4.1 D.).

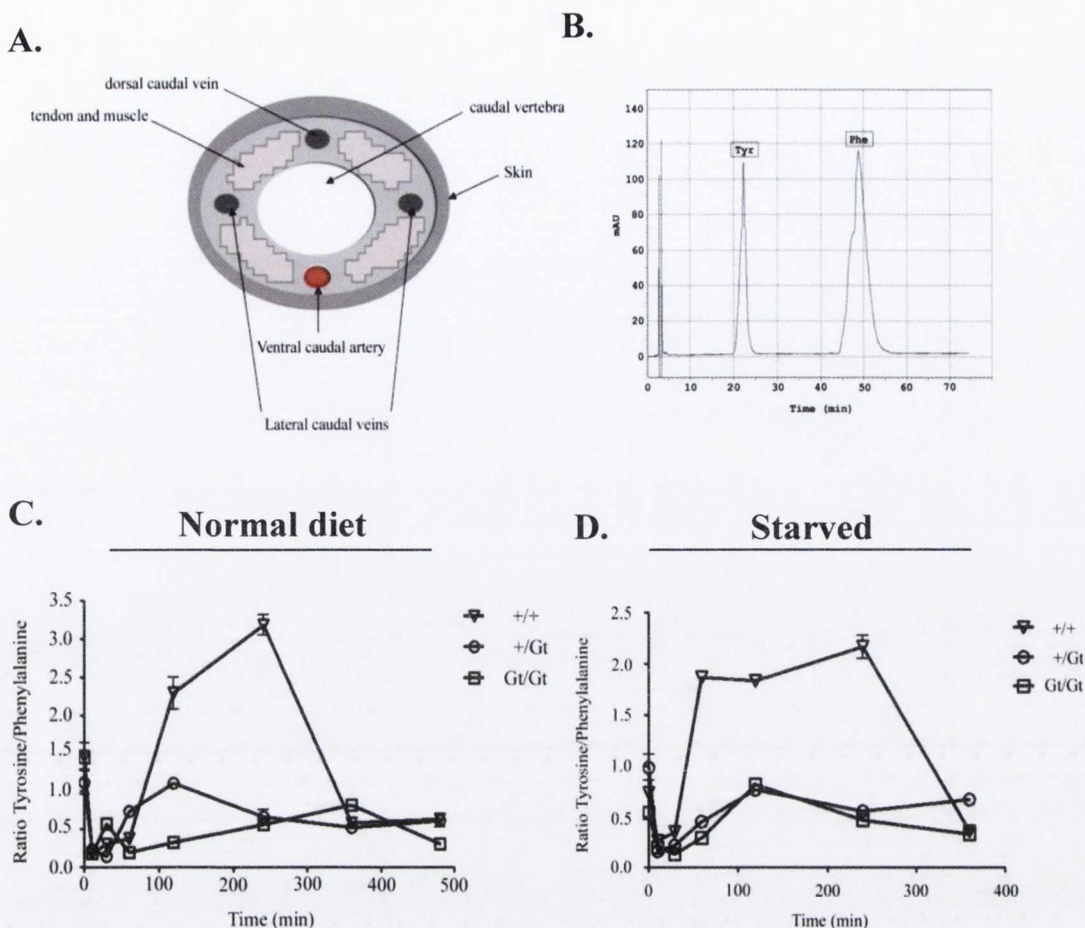


Figure 4.2.1 Phenylalanine to tyrosine conversion is decreased in *Qtrt1*^{+/-Gt} and *Qtrt1*^{Gt/Gt} mice relative to wild-type animals. (A) Schematic representation of a mouse tail transverse section showing the location of veins and arteries. The ventral caudal artery was used for arterial blood collection (*adapt.* Argmann CA *et al.*, 2006). (B) Elution profile of L-tyrosine (Tyr) and L-phenylalanine (Phe) standards following reversed-phase separation on a Zorbax 300SB-C18 column at a flow rate of 1 ml/min. Tyrosine was detected by the absorbance at 206nm. The plasma phenylalanine to tyrosine conversion ratio following intraperitoneal injection (IP) of phenylalanine (1mg/g body weight) in wild-type (+/+), heterozygous (+/-Gt) and homozygous (Gt/Gt) TGT gene-trap mice maintained on normal diet (C) or starved for 12 hours (D).

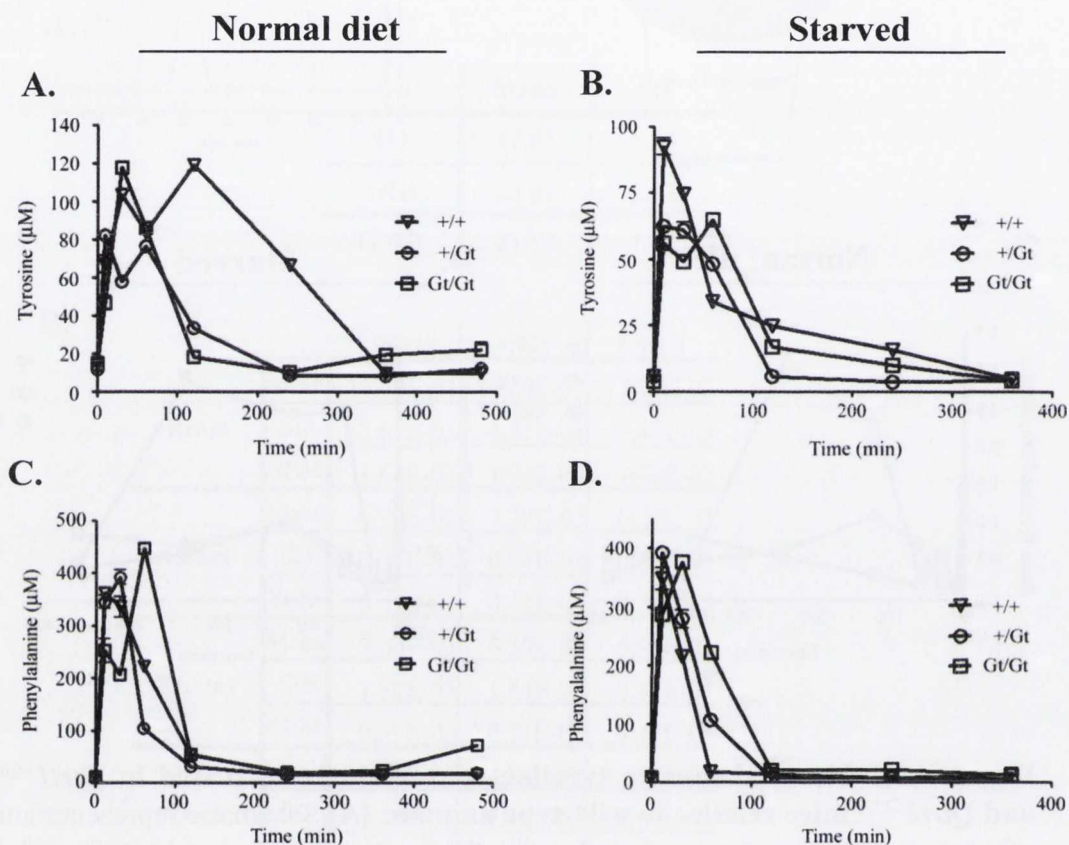


Figure 4.2.2 Assay of phenylalanine to tyrosine conversion in wild-type, *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice. Plasma tyrosine concentrations following intraperitoneal injection (IP) of phenylalanine (1mg/g body weight) to wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene-trap mice maintained on normal diet (A) or starved for 12 hours (B). Similarly, plasma phenylalanine concentrations were determined for mice maintained on a normal diet (C) or starved for 12 hours (D).

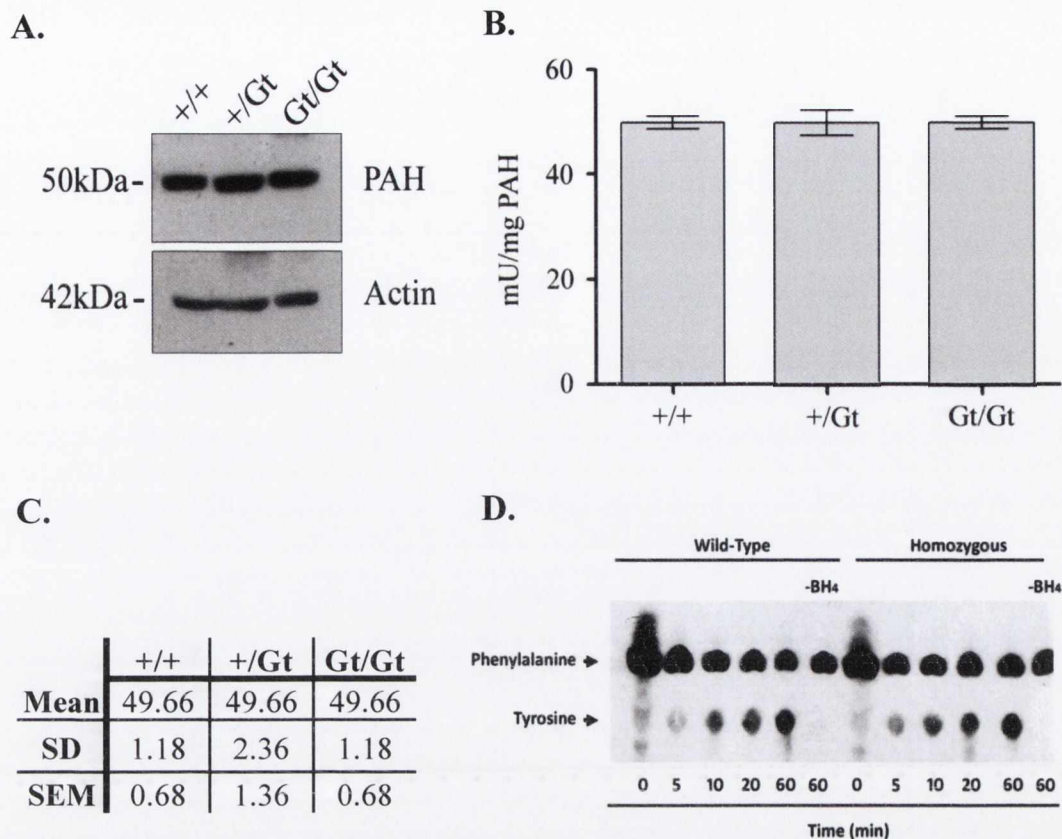


Figure 4.3 Liver phenylalanine hydroxylase protein level and enzymatic activity is unaltered in *Qirt1*^{+/Gt} and *Qirt1*^{Gt/Gt} mice relative to wild-type controls. (A) Western blot of liver PAH levels in liver cytosolic extracts (4μg) from wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) mice. The blot was probed with PAH specific antisera raised against recombinant protein. (B) Kinetic analysis of mouse PAH protein in liver cytosolic extracts from wild-type, heterozygous and homozygous mice. PAH activity assay was carried out in the presence of 100μM BH₄ (Figure 4.3 B.). (C) Statistical analysis of data obtained in PAH activity assay (D) Measurement of PAH protein activity in liver homogenates of wild-type, heterozygous and homozygous TGT genetrap mice. Undiluted liver cytosol (18μl) was incubated for varying times with ¹⁴C-phenylalanine and tetrahydrobiopterin. The ¹⁴C-tyrosine produced was resolved from ¹⁴C-phenylalanine by thin layer chromatography (TLC) and visualised by autoradiography.

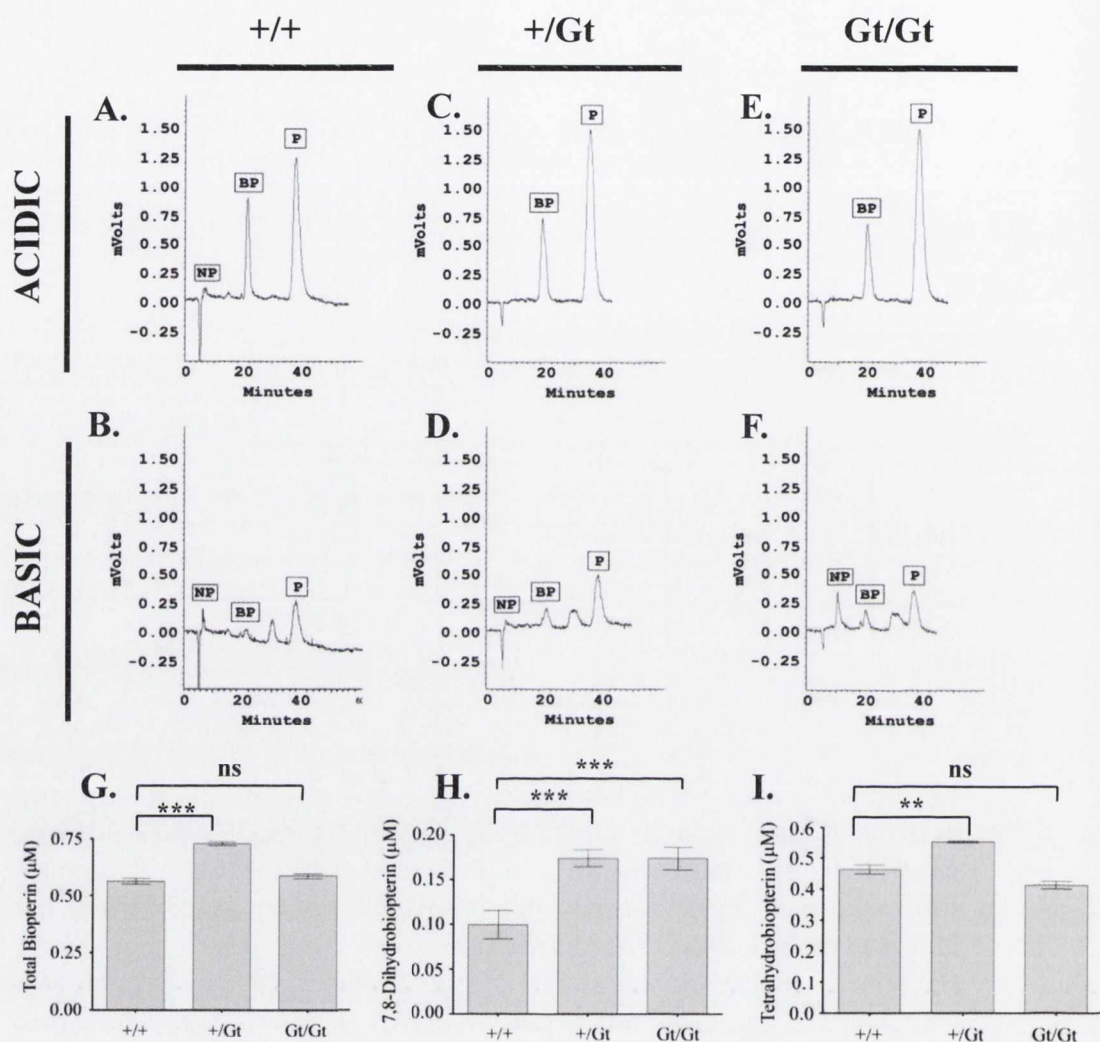


Figure 4.4 Liver 7,8 dihydrobiopterin levels are increased in *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt/Gt*} mice relative to wild-type animals. Representative HPLC chromatographs of pterins in cytosolic liver fractions from wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) mice following acidic (A, C, E) and basic (B, D, F) iodine oxidation. Reversed-phase separation was carried out on a Spherisorb ODS-1 column eluted with 5% methanol - 95% water at 0.6ml/min. Pterins were detected by their native fluorescence (λ_{ex} =350nm and λ_{em} =450nm). Total biopterin (G), 7, 8 dihydrobiopterin (H) and tetrahydrobiopterin (I) levels in liver cytosolic fractions of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT genetrapped mice. Each bar represents the mean value $\pm$ SD of 3 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t-test. (See Table 4.2 for Mean values, SD and SEM)

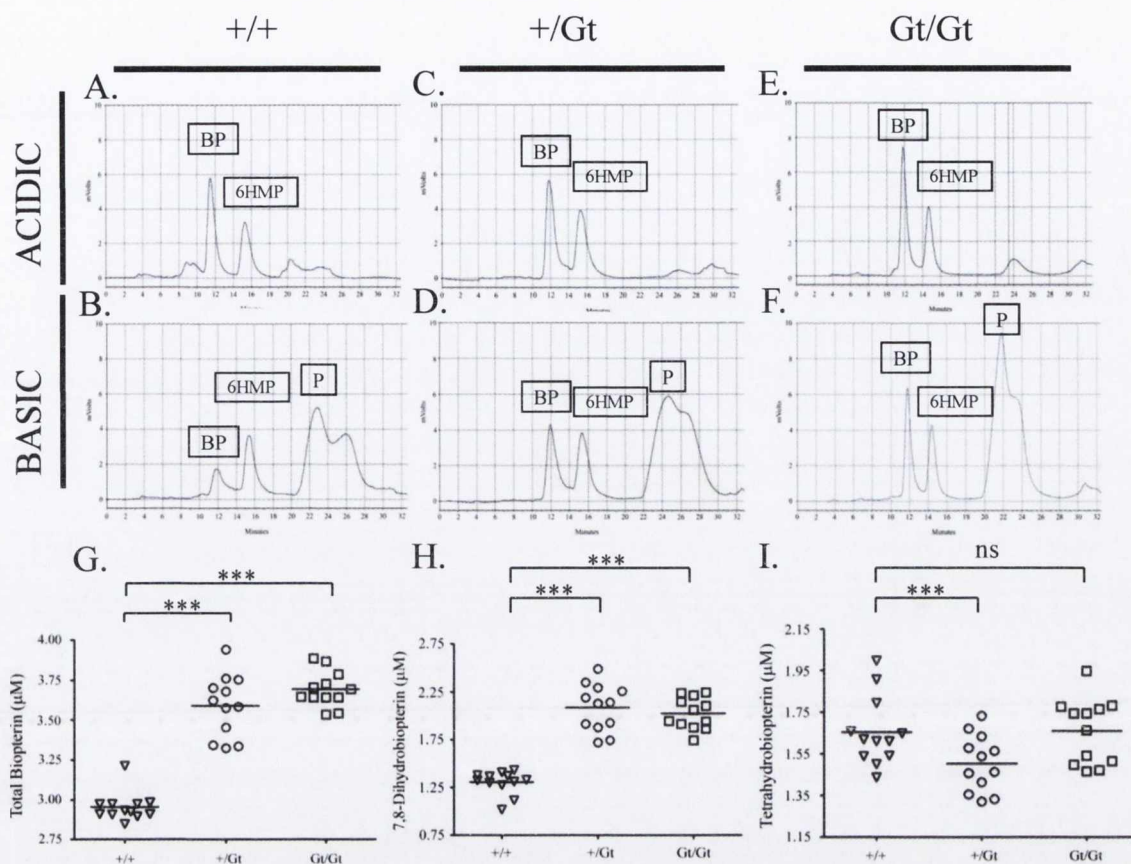


Figure 4.5 Total biopterin and 7,8 dihydrobiopterin levels are increased in the urine of *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt/Gt*} mice relative to wild-type control animals. Representative HPLC chromatographs of pterins in the urine of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice following acidic (A, C, E) and basic (B, D, F) iodine oxidation. Reversed-phase separation was carried out on a Spherisorb ODS-1 column eluted with 5% methanol - 95% water at 0.6ml/min. Pterins were detected by their native fluorescence (λ_{ex} =350nm and λ_{em} =450nm). Graphic representation of the total biopterin (G), 7, 8 dihydrobiopterin (H) and tetrahydrobiopterin (I) levels in the urine of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT genetrapped mice. Each point represents the average value from triplicate measurements in one mouse. *P<0.05, **P<0.01, ***P<0.001, Student's t-test. (See Table 4.2 for Mean values, SD and SEM)

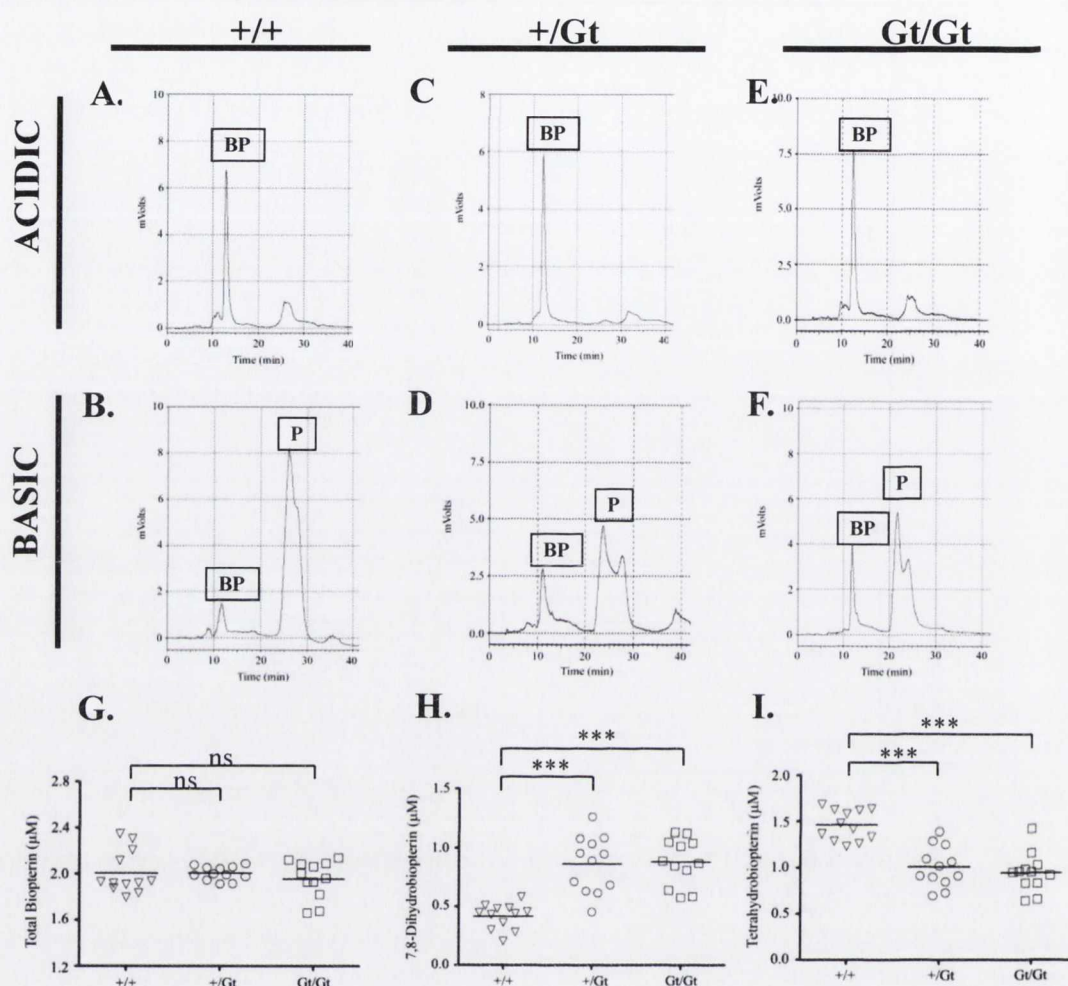
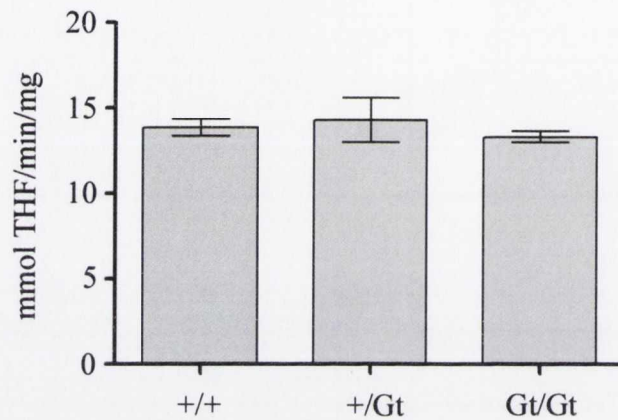


Figure 4.6 7,8 dihydrobiopterin levels are increased whereas tetrahydrobiopterin levels are decreased in the serum of *Qtrt1*^{+/-Gt} and *Qtrt1*^{Gt/Gt} mice relative to wild-type control animals. Representative HPLC chromatographs of pterins in the urine of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice following acidic (A, C, E) and basic (B, D, F) iodine oxidation. Reversed-phase separation was carried out on a Spherisorb ODS-1 column eluted with 5% methanol - 95% water at 0.6ml/min. Pterins were detected by their native fluorescence (λ_{ex} =350nm and λ_{em} =450nm). Graphic representation of the total bipterin (G), 7, 8 dihydrobiopterin (H) and tetrahydrobiopterin (I) levels in the urine of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT genetrapp mice. Each point represents the average value from triplicate measurements in one mouse. *P<0.05, **P<0.01, ***P<0.001, Student's t-test. (See Table 4.2 for Mean values, SD and SEM)

			+/+	+/Gt	Gt/Gt
Liver	Total Biopterin	Mean	0.56	0.73	0.59
		SD	13.92E-03	6.85E-03	10.58E-03
		SEM	8.04E-03	3.95E-03	6.11E-03
	Tetrahydrobiopterin	Mean	0.46	0.55	0.41
		SD	26.17E-03	3.37	20.48E-03
		SEM	15.11E-03	1.94E-03	11.83E-03
	Dihydrobiopterin	Mean	0.10	0.17	0.17
		SD	15.58E-03	9.68E-03	12.63E-03
		SEM	8.99E-03	5.59E-03	7.29E-03
Urine	Total Biopterin	Mean	2.953	3.589	3.692
		SD	0.09	0.19	0.11
		SEM	26.20E-03	56.15E-03	32.04E-03
	Tetrahydrobiopterin	Mean	1.656	1.505	1.66
		SD	0.17	0.14	0.16
		SEM	47.99E-03	39.49E-03	45.58E-03
	Dihydrobiopterin	Mean	1.301	2.084	2.025
		SD	0.12	0.25	0.16
		SEM	35.20E-03	70.90E-03	47.67E-03
Plasma	Total Biopterin	Mean	2.01	2.01	1.96
		SD	0.19	0.06	0.16
		SEM	54.02E-03	17.66E-03	47.31E-03
	Tetrahydrobiopterin	Mean	0.42	0.86	0.88
		SD	0.11	0.24	0.20
		SEM	30.91E-03	69.42E-03	57.51E-03
	Dihydrobiopterin	Mean	1.46	1.01	0.95
		SD	0.16	0.22	0.21
		SEM	47.35E-03	63.24E-03	60.56E-03

Table 4.2. Analysis of data obtained during measurement of biopterin levels in liver, urine and plasma. Statistical analysis of total biopterin, tetrahydrobiopterin and dihydrobiopterin in liver (Figure 4.4), urine (Figure 4.5) and plasma. (Figure 4.6).

A.



B.

	+/+	+/Gt	Gt/Gt
Mean	13.82	14.31	13.33
SD	0.49	1.30	0.32
SEM	0.28	0.75	0.19

C.

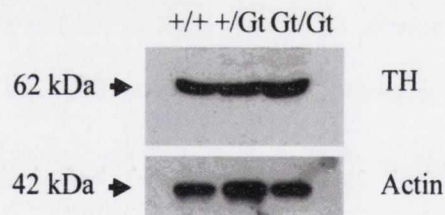


Figure 4.7 DHFR activity and level of TH expression in liver of wild-type and TGT genetrap mice. **A)** Kinetic analysis of mouse DHFR protein in wild-type, heterozygous and homozygous mice. The rate of reaction of DHFR enzyme was measured in liver homogenate. **B)** Statistical analysis of DHFR activity in liver of wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) mice (Figure 4.7 A.). **C)** Western blot analysis of liver TH levels in three genotypes of TGT knockout mice. Cytosolic liver protein (4 μ g) was extracted from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice, resolved on a SDS-PAGE gel and transferred to a PVDF transfer membrane. The blot was probed with TH specific antisera and equal levels of TH protein were found in all three genotypes.

4.3 Discussion

The characterization of TGT gene-trap mice is crucial to understanding the role of queuosine in living organisms. The results obtained correlate with the data obtained by Reyniers *et al.* [39]; that the absence of queuosine modification from tRNA has no obvious adverse effect on mice under laboratory conditions. Wild-type and gene trap mice were similar in appearance, and although there appeared to be some increase in their body weight, only spleen showed a notable decrease in weight.

One of the most important differences that was found in these mice was that tyrosine biosynthesis is compromised over time in both heterozygous and homozygous TGT gene-trap mice after exogenous administration of phenylalanine. The expression and activity of PAH was found to be the same in wild-type and TGT gene-trap mice, ruling out the possibility that PAH is responsible for the defect. As would be expected for animals that have difficulty in producing tyrosine, the administration of phenylalanine lead to its accumulation over time to a greater extent in gene trap mice relative to wild-type animals. Interestingly, within the first 30 minutes tyrosine production appeared to be near normal levels in gene-trap mice then dramatically dropped off. This phenomenon most likely arises from a drop in reduced tetrahydrobiopterin, given that the PAH levels and activity were normal. It might be useful in future work to carry out a tracer experiment, such as administering ^{14}C -U-phenylalanine and then measuring the radioactive tyrosine produced, in order to establish unequivocally that the tyrosine measured was derived from the phenylalanine added.

It is interesting that tyrosine biosynthesis is found to be compromised over time in heterozygous mice, since one would expect that they should have half-normal TGT activity due to the presence of one functional allele, and hence have the ability to form queuosine-tRNA. However, in our laboratory Coilin Boland demonstrated that heterozygous animals at 6-8 weeks of age appear to have no queuosine in their tRNA and 12 to 18 weeks old mice do. The animals that were used for the purposes of this study were all 6 to 8 weeks old. It is worth highlighting that animals are born germ-free (axenic) and do not have queuosine modified tRNA. It is highly likely that heterozygous animals require a longer period of time to produce detectable levels of modified tRNA. A second reason for the slow accumulation of queuosine tRNA could be the product of the gene trap allele, an inactive fusion protein, acting as a dominant negative inhibitor,

sequestering away the TGT partner protein Qv1 away from the normal wild-type TGT protein.

In some cases of PKU, high phenylalanine levels are caused by a defect in either the salvage or regeneration pathways of BH₄ [91]. Since tyrosine production is affected in our TGT genetrap mice and this deficit is not due to PAH protein the next logical step was the measurement of BH₄. The most significant change occurred in the level of BH₂, which was found to be high in liver, plasma and urine of TGT genetrap mice when compared to wild-type. Total biopterin was also found to increase in urine and tetrahydrobiopterin was found to be low in plasma of TGT genetrap mice when compared to wild-type.

In the process of BH₄ regeneration, quinonoid dihydrobiopterin is formed, which is required by DHPR enzyme. However, it is an extremely unstable compound and is readily tautomerized to BH₂. The deficiency in DHPR can therefore lead to accumulation of BH₂. The BH₄ deficiency due to defective DHPR enzyme has been shown to cause atypical phenylketonuria or hyperphenylalaninemia [158-159]. Literature search did not yield information on whether BH₂ has inhibitory effect of PAH protein. The BH₂ can also be utilized by DHFR in a salvage pathway to regenerate BH₄ however the accumulation of BH₂ is not due to the inactivity of this enzyme.

It was also previously shown that the level of BH₂ is high at the onset of diabetes and other chronic vaso-inflammatory conditions [121]. The ratio of BH₄/BH₂ was also found to be low in the plasma of patients with cardiovascular disorders [122-124] and with insulin resistance [123]. It was suggested that uncoupling of the NOS reaction could be promoting the oxidation of BH₄ to BH₂ which stimulate production of superoxide and peroxynitrite [135]. Interestingly, superoxide formation is found in patients with deficiency in BH₄ metabolism [132-134].

In light of the changes in BH₂, this could provide yet another explanation for the slow accumulation of queuosine modified tRNA in heterozygous animals. Biopterin sepiapterin, tetrahydrobiopterin and other pteridine analogues have previously been shown to inhibit purified queuine tRNA ribosyltransferase activity [160]. Therefore, it is also quite likely that BH₂ may also act as an inhibitor.

In conclusion, given the changes in tyrosine biosynthesis over time, BH_4 and BH_2 there is a strong possibility that TGT genetrapped mice will display further characteristics of PKU and BH_4 disorders. These will be examined in subsequent chapters.

Chapter 5

Q-tRNA leads to behavioural changes in female TGT genetrap mice

5.1 Introduction

The BH₄ has been implicated in many diverse biological functions. It has been shown to react readily with O₂ to produce free radicals [104-105] resulting in the formation of oxidative species such as superoxide, H₂O₂, and peroxinitrite[161]. Conversely, it has also been shown to protect cells against oxidative damage by scavenging free radicals [162]. The BH₄ seems to possess proliferative activity in hemopoietic cells [104-105] and in conjunction with sepiapterin it was also found to enhance proliferation of SV40-transformed human fibroblasts, rat C6 glioma cells and PC12 cells [106-107]. The BH₄ was shown to reduce NO-induced endothelial cell death, possibly by scavenging H₂O₂ [163]. In another study however it was suggested that the increase in BH₄ during ischemia plays an important role in selective neuronal injury via NOS activation [164].

There many different disorders associated with BH₄ deficiencies, which can be divided into two major categories, monoamine neurotransmitter deficiencies with hyperphenylalaninemia (classical form of BH₄ deficiency) and monoamine neurotransmitter deficiencies without hyperphenylalaninemia (DRD and SR deficiencies) [165]. Several neurological diseases are also known to have a deficiency in BH₄ such as Parkinson's disease [166]. Alzheimer's disease [167], autism [168] and depression[169].

Mutations that affect BH₄ gives rise to phenylketonuria, characterized by high levels of phenylalanine in the blood. The accumulation of phenylalanine had been shown to interfere with normal development of central nervous system, leading to microcephaly [170-171]. The progression of this disease can result in mental retardation and other neurological disorders if it is left untreated [76-78]. Adult patients with phenylketonuria who failed to be treated during their infancy—before mandatory newborn screening came into effect—have symptoms including seizures, neurological disorders, eczema, muscle hypertonicity and behavioural disorders such as hyperactivity, aggressive and erratic behaviours, motor and attention disturbances, negative mood swings, irritability, night terrors and self-inflicted injury [172-173]

The BH₄ acts as a cofactor for all aromatic amino acid hydroxylases (phenylalanine, tyrosine and tryptophan hydroxylases). Therefore, a defect in the biosynthetic and regeneration pathways of BH₄ can affect the production of monoamine neurotransmitters in addition to tyrosine. This can result in the development of neurological and behavioural problems [73, 174]. Maintaining healthy levels of BH₄ in the brain is therefore crucial for proper brain function.

In the previous chapter it was shown that queuosine-tRNA is necessary for maintaining BH₄ levels as well as tyrosine production from phenylalanine over time. The possibility exists that queuine is involved in BH₄ recycling or in the protection against the oxidative modification of tetrahydrobiopterin to BH₂. It would be interesting to know if enzymes responsible for queuosine modification of tRNA in eukaryotes are expressed in the brain and to what extent. Recent studies in our laboratory [36] showed that queuosine formation of eukaryotic tRNA occurs via a mitochondrial-localized TGT-Qv1 complex, with Qv1 being the predominant splice variant of the QTRTD1 gene in adults. The investigation into the expression levels of these two genes could be carried out by looking at the Allen Mouse Brain Atlas (<http://www.brain-map.org/>) which is an interactive genome-wide image database of gene expression profiles.

Mice have a distinct advantage over other experimental species (i.e. worms, fly, yeast) when investigating physiological function as they are genetically and anatomically more closely related to humans. They are increasingly used to look at the *in vivo* function of molecules that have been already studied *in vitro*. The studies carried out with these mice can be on the levels of biochemistry, cell biology, physiology and behaviour. Complex behavioural changes can be observed with mice that have a single gene alteration [175]. Creating a knockout mouse can help to understand how one protein can contribute to a change in behaviour. Behavioural tests aid in determination of neurological health of a transgenic animal providing that proper controls are set up [176].

One of the simplest behavioural tests to be carried out is one for locomotion and movement. This test is used as a control experiment for more complex behavioural tests. Locomotion is usually measured as novelty and exploratory behaviour, in response to drug treatment or just as an unstimulated activity. Several of the brain systems such as the telencephalic dopaminergic system and the cerebellum, as well as abnormalities in the periphery such as muscle weakness or motoneuron degeneration can have an affect on

locomotion. Locomotion can also be affected by the ill health of the animal [175]. Several studies have shown that defects in dopamine synthesis enzymes, receptors and transporters results in changes in locomotion [177-179]. Mice that are a deficiency in dopamine are severely hypoactive, while disruption in dopamine transporter activity results in hyperactivity [177].

The behaviour of mice is usually tested in either open field or in their home cage environment. However, it is often uncertain whether differences in behaviour arise from changes in the level of anxiety or from differences in locomotor activity [180-181]. When tested in an open field setting, mice can display several behavioural components such as novelty seeking, fear responses, stereotypy and reaction to being handled [182-183]. Despite this, locomotion in the open field is often used in order to look at general locomotor activity or exploratory behaviour [184-185] and anxiety [186-187]. Behavioural testing carried out in a home cage environment allows assessment of the animal's activity with minimal external influences that are usually associated with open field testing. By combining open cage and home cage test results it is possible to distinguish between spontaneous activities exhibited in familiar surroundings and in a novel environment.

In 1972 a paper was published by Andersen *et al.* [188] in which behavioural changes were studied in rats with experimental phenylketonuria. In this model the activity of phenylalanine hydroxylase enzyme was inhibited by *p*-chlorophenylalanine and rats were supplemented with phenylalanine to produce high ratio of phenylalanine to tyrosine in the blood. It was noted that rats treated in such a way were more active in the open field experiment than the controls. This coincided with a clinical characteristic of hyperactivity in PKU patients. Several other symptoms displayed suggested that rats with experimental phenylketonuria can be used to study this disease [188].

5.2 Results

5.2.1 Expression levels of TGT and Qv1 in mouse brain

Queuosine modification of tRNA in eukaryotes is carried out by the TGT enzyme in association with a mitochondrial-localised Qv1 protein. In this part of the study it was decided to look at the mRNA levels of these two proteins in the mouse brain.

The Allen institute for brain science provides a mouse brain atlas, which is an interactive and genome-wide image database of gene expression (<http://mouse.brain-map.org/welcome.do>). It was possible to retrieve and analyse *in situ* hybridization (ISH) images of TGT and Qv1 expression from this database. The ISH imaging shows the cellular localization of mRNA transcripts for specific genes. Antisense probes are labelled specifically for a particular gene and are hybridized to cellular transcripts. The detection of the bound probe then produces specific labelling in cells that express this particular gene. The use of alkaline phosphatase substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) allows the colorimetric detection of this bound probe due to formation of blue/purple reaction product.

ISH of TGT and Qv1 was examined in mouse brain sagittal sections (Figure 5.1 A, B and C). The ISH images shown here are of three different slices with their position indicated by the left most images. The expression level of TGT and Qv1 in specific brain regions was also graphed to allow comparison of the relative expression of these two proteins in different brain regions (Figure 5.1 D). From this data it is apparent that TGT transcript is predominantly expressed in the Cereberum and Limbic region of the brain while the expression of Qv1 is found to be highest in the Posterior Cortex.

5.2.2 Open field activity of wild-type, *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt*/*Gt*} mice

The exploratory activity of wild-type and TGT genetrapped mice was measured with the aid of an EthoVision Video Tracking System. Figure 5.2 shows the set up of this experiment. A video camera takes behavioural recordings from the exploratory box apparatus consisting of one circular arena (71 cm in diameter). This video recording is then analysed by computer frame-by-frame by EthoVision Software which can distinguish mice from the background, on the basis of their darker colour (Figure 5.2 A).

After tracking by EthoVision software, the position in the open field was analysed for eight female and eight male mice on a wild-type, *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} background. During this analysis the arena was divided into two zones, an inner and outer zone (Figure 5.2 B). This type of open field testing allowed measurement of anxiety-related behaviour. When mice are anxious they tend to stay close to the walls, i.e. in the outer zone, a behaviour response that is referred to as thigmotaxis. The animals that are less anxious spend more time in the inner zone. The experiment was carried out for a total duration of 30 minutes. However, by analysing the data with Ethovision it was possible to divide the results obtained into the initial 10 minutes and the last 20 minutes. The total duration of time, first 10 minutes and last 20 minutes spent in inner and outer zone were then separately plotted and analyzed for female (Figure 5.3, (See Table 5.1 for Mean values, SD and SEM)) and male mice (Figure 5.4, (See Table 5.2 for Mean values, SD and SEM)).

The results obtained for female mice show a significant difference in the total time spent in inner ($p < 0.01$) and outer ($p < 0.05$) zones for *Qtrt1*^{Gt/Gt} mice when compared to wild-type mice (Figure 5.3). Homozygous female mice seem to spend more time in the inner zone than the outer zone. However, this difference is not present in the initial first 10 minutes, but appears only in the last 20 minutes ($p < 0.05$). This difference was not observed in female *Qtrt1*^{+/Gt} mice. The results obtained for male mice (Figure 5.4) showed absolutely no difference between the three genotypes.

In conclusion, this data showed that female *Qtrt1*^{Gt/Gt} mice are less anxious and ready to explore new terrain in a much faster time than wild-type or *Qtrt1*^{+/Gt} mice. Surprisingly, this change in behaviour is limited to females and does not occur in male *Qtrt1*^{Gt/Gt} mice.

5.2.3 Home cage activity of wild-type, *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice

In order to assess the behaviour of mice with only minimal external influences home cage activity testing was carried out. Female and male mice ($n=8$ for each gender) were individually placed in a standard plastic-bottomed cage equipped with a home cage activity monitor. Behaviour was then recorded for the duration of an hour with passive infrared sensor mounted above the floor cage (Figure 5.5). Exploratory and movement behaviours were then recorded in the form of activity counts obtained from interruption

of sensor beams. The behaviour was quantified by static, mobile, rearing and total mobile time for female and male mice taken at 1 minute intervals for the duration of one hour (Figure 5.6).

A difference was observed in static counts for female mice, especially between 18 and 45 minutes. $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ mice were found to be more stationary at the time that these measurements were taken when compared to wild-type (Figure 5.6 A). No difference in static counts was observed for male mice (Figure 5.6 B). $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ female mice also exhibited a higher level of mobility quantified by mobile counts, in the initial half an hour when compared to wild-type mice (Figure 5.6 C). Again no difference was observed in male mice (Figure 5.6D). The analysis of rearing counts showed that in the first half an hour as well as being more mobile $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ female mice reared more when compared to female wild-type mice (Figure 5.6 E.). No difference in the number of rearings were observed for male mice (Figure 5.6 F.). The total time that $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ female mice were found to be mobile was also found to be higher in the first 30 minutes of the testing (Figure 5.6 G). While male mice again displayed no difference (Figure 5.6 H).

From these results it can be concluded that only female TGT genetrap mice exhibit behavioural differences. In the initial half an hour $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ female mice were more active when compare to wild-type. Behavioural changes observed in $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ female mice in the initial 30 minutes consisted of constant movement with sniffing of the floor and walls of the cages and a lot of rearing. In contrast, wild-type mice exhibited the usual exploratory sniffing of wild-type mice which was more irregular, not continuous and had few rearings.

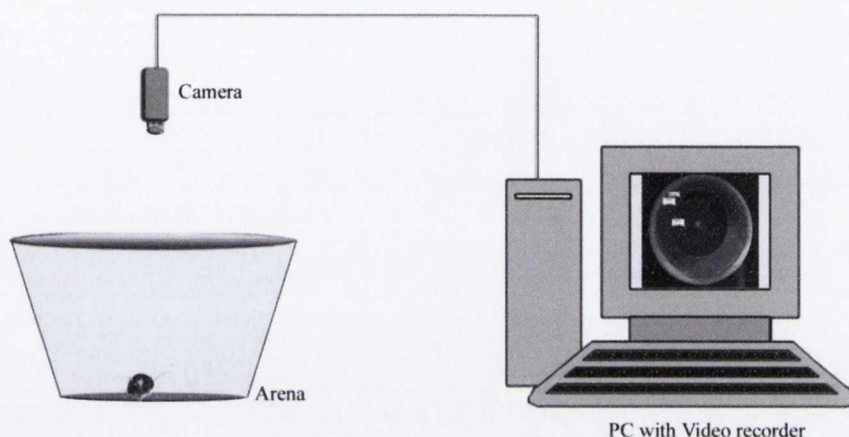
The total number of counts were then calculated per animal and the graphs representing static, mobile, rearing and total mobile time behaviour were plotted (Figure 5.7, (See Table 5.3 for Mean values, SD and SEM)). Female $Qtrt1^{Gt/Gt}$ mice showed significantly higher activity ($p<0.001$) than the $Qtrt1^{+/Gt}$ and wild-type female mice. They were found to be more mobile (Figure 5.7 C) for longer periods of time (Figure 5.7 G) and to rear at a much higher frequency (Figure 5.7 E). Interestingly, when the total number of static counts was calculated it was found to be higher in $Qtrt1^{Gt/Gt}$ female mice when compared to female $Qtrt1^{+/Gt}$ and wild-type mice (Figure 5.7 A). However, this can be explained by looking at the number of counts measured per minute for the duration of

one hour (Figure 5.6 A). In that figure an increase in static counts was observed for homozygous female mice between approx. 18 to 45 minutes. This accounts for the increase in total number of stationary counts. Surprisingly, no significant difference was observed in wild-type, *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} male mice (Figures 5.7 B, D, F and H.).

In the process of carrying out these behavioural experiments excessive grooming behaviour in *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice was noted when compared to wild-type mice. So it was decided to calculate total time spent grooming per hour for each of the genotypes. The total time spent grooming was recorded manually for each individual female (Figure 5.8 A, (See Figure 5.8 E for Mean values, SD and SEM)) and male mouse (Figure 5.8 B, (See Figure 5.8 E for Mean values, SD and SEM)). The analysis of the data obtained showed significant increase ($p < 0.001$) in the time spent grooming for *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} female mice. No significant difference was observed in *Qtrt1*^{Gt/Gt} male mice.

A separate rating scale was also introduced for quantification of stereotypy behaviour of TGT genetrapped mice. This measure of stereotypy behaviour was based on time sampling and observational recordings. The behaviour observed was recorded every 5 minutes for the duration of one hour. The resultant graphs comparing stereotypy in wild-type *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} female and male mice showed significant difference in behaviour of TGT genetrapped mice for both genders (Figures 5.8 C and D respectively). The behavioural differences such as extensive grooming, constant locomotion, high frequency of rearing, constant sniffing while moving of air, floor and walls of the cage and continuous sniffing in one spot were noted. These behavioural differences were observed at a higher degree in female TGT genetrapped mice than male.

A.



B.

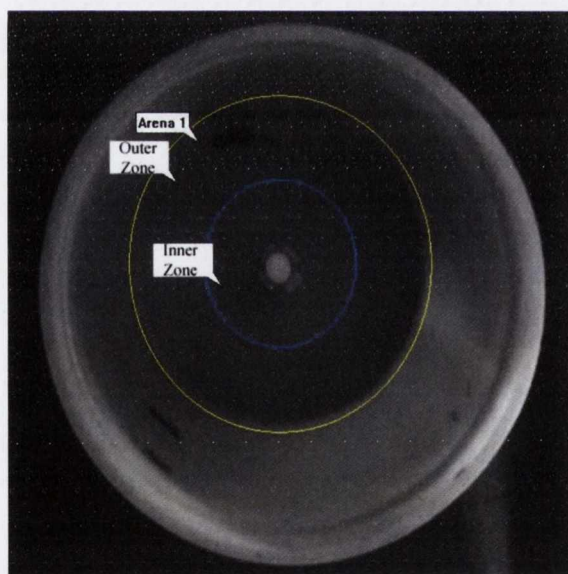


Figure 5.2 Schematic representation of the Ethovision set-up. (A) A camera captures an analog version of the image which is fed into a frame grabber on the computer in order to digitize the image. The EthoVision program then analyzes the signal to produce a quantitative description of mouse behaviour. (B) Photographic representation of the experimental set up with arena and different zones. The circular arena (a region from which data is acquired, here labelled Arena) is divided into two zones, the outer zone and the inner zone.

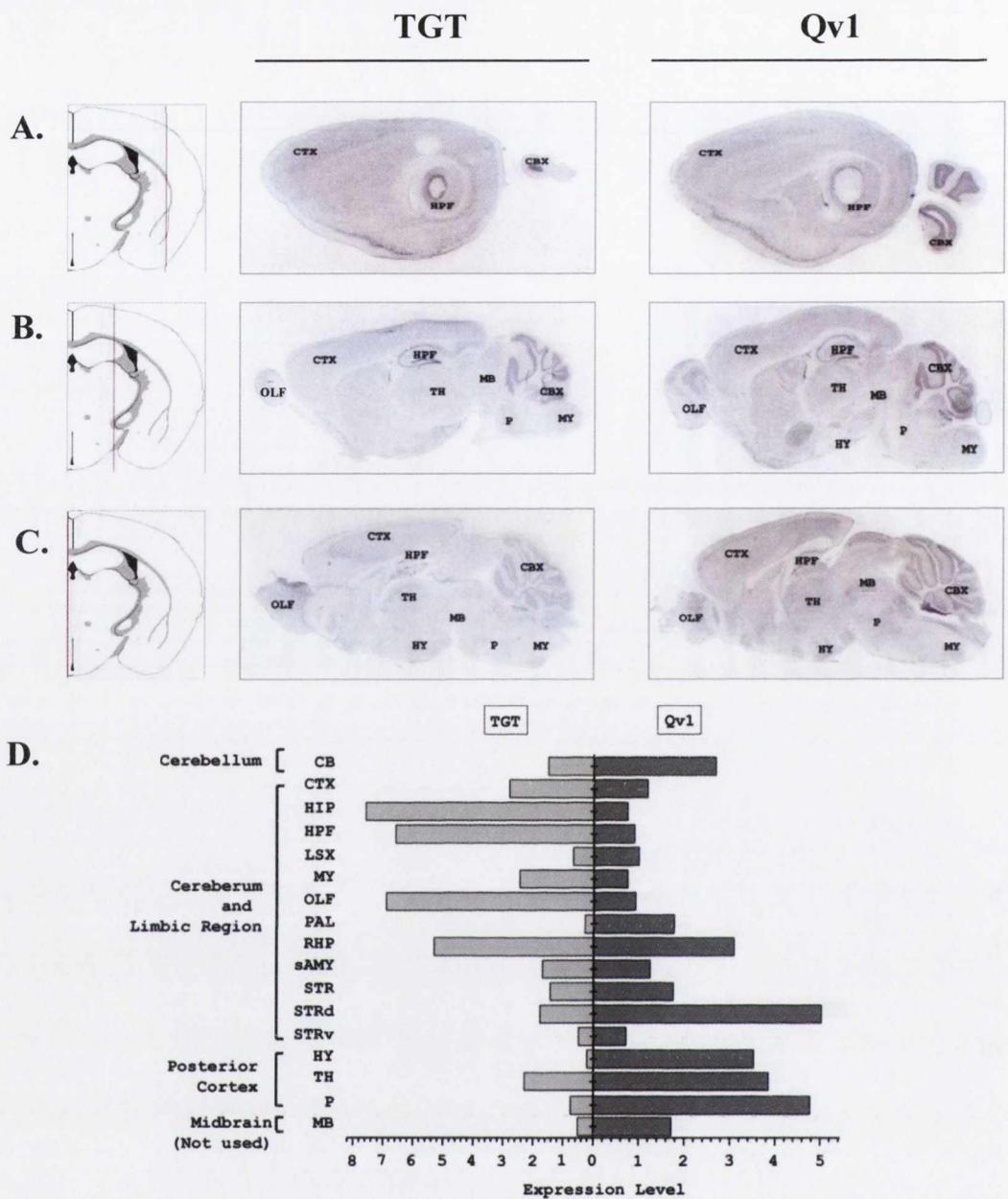


Figure 5.1 *In situ* hybridization of TGT and Qv1 in mouse brain sagittal sections from the *Allen mouse brain atlas*. C57BL/6J male mice (aged 56 days, with a body weight of 25.10 $\pm$ 1.59g) were used for *in situ* hybridization (ISH) of brain sagittal sections (25 μ m). ISH images (A), (B) and (C) show the expression of TGT and Qv1 from three different sections through the brain. The specific brain regions are labelled as follows: cerebral cortex (CTX), hippocampal formation (HPF), cerebellar cortex (CBX), medulla (MY), pons (P), midbrain (MB), thalamus (TH), olfactory bulb (OLF) and hypothalamus (HY). (D) Graph showing the relative expression levels of TGT and Qv1 in different brain regions.

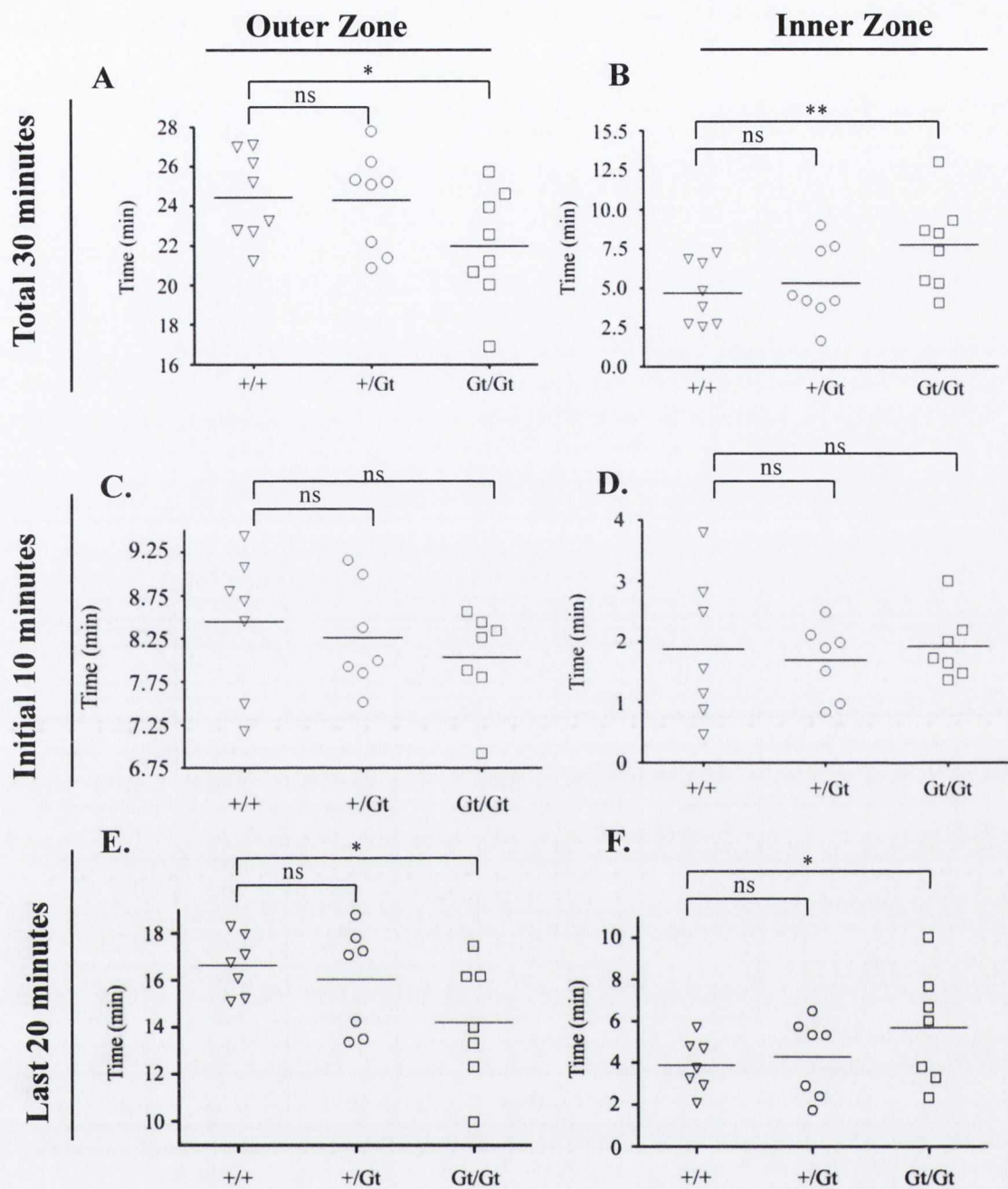


Figure 5.3 Open field analysis of female wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Time spent by female mice in the outer (A, C, E) and inner zone (B, D, F) of the open-field test for the total 30 minutes (A, B), the initial 10 minutes (C, D) and the last 20 minutes (E, F) of the experiment. Statistical analysis was carried out using the Student t-test (*P<0.05, **P<0.01, ***P<0.001). (See Table 5.1 for Mean values, SD and SEM)

		Outer Total			Inner Total		
		+/+	+/Gt	Gt/Gt	+/+	+/Gt	Gt
Total	Mean	24.41	24.27	21.96	4.65	5.3	7.72
	SD	2.23	2.48	2.85	2.01	2.44	2.83
	SEM	0.79	0.88	1.00	0.71	0.86	1.00
Initial 10 minutes	Mean	8.15	8.15	8.03	1.79	1.79	1.92
	SD	1.12	0.65	0.56	1.13	0.62	0.55
	SEM	0.40	0.23	0.21	0.40	0.22	0.21
Last 20 minutes	Mean	16.30	15.68	14.20	3.58	3.84	5.69
	SD	1.49	2.27	2.59	1.43	2.15	2.71
	SEM	0.53	0.80	0.98	0.51	0.76	1.02

Table 5.1. Analysis of data obtained during open field experiment with female mice. Statistical analysis of data obtained during measurement of open field activity of female wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 5.3).

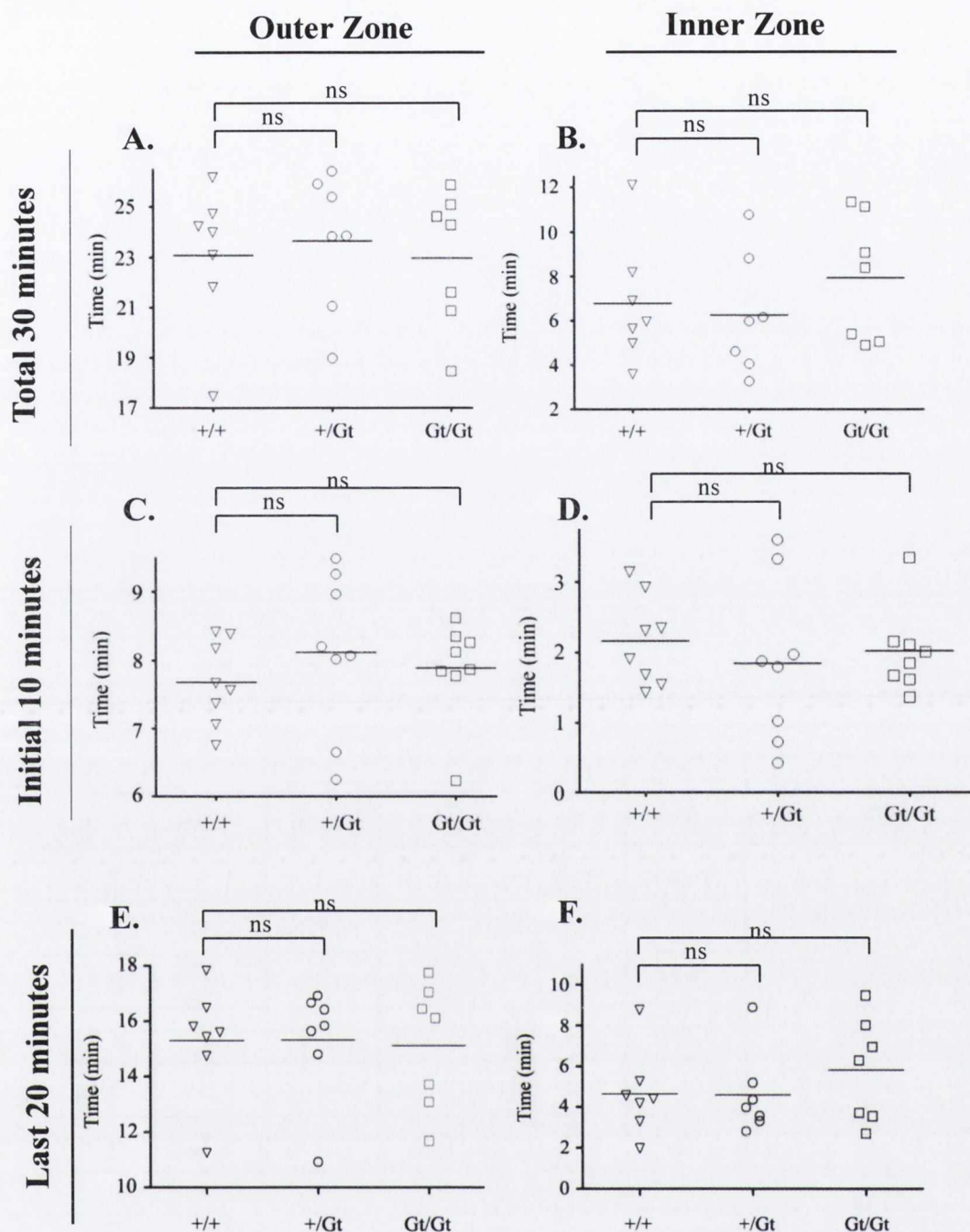


Figure 5.4 Open field analysis of male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Time spent by male mice in the outer (A, C, E) and inner zone (B, D, F) of the open-field test for the total 30 minutes (A, B), the initial 10 minutes (C, D) and the last 20 minutes (E, F) of the experiment. Statistical analysis was carried out using the Student t-test (*P<0.05, **P<0.01, ***P<0.001). (See Table 5.2 for Mean values, SD and SEM)

		Outer Total			Inner Total		
		+/+	+/ <i>Gt</i>	<i>Gt/Gt</i>	+/+	+/ <i>Gt</i>	<i>Gt</i>
Total	Mean	23.07	23.63	22.96	6.76	6.23	7.91
	SD	2.82	2.72	2.71	2.77	2.69	2.82
	SEM	1.06	1.03	1.02	1.05	1.02	1.06
Initial 10 minutes	Mean	7.67	8.11	7.88	2.16	1.84	2.02
	SD	0.61	1.17	0.73	0.64	1.15	0.60
	SEM	0.22	0.41	0.26	0.22	0.41	0.21
Last 20 minutes	Mean	15.30	15.31	15.12	4.64	4.60	5.81
	SD	2.04	2.07	2.28	2.10	2.04	2.54
	SEM	0.77	0.78	0.86	0.79	0.77	0.96

Table 5.2. Analysis of data obtained during open field experiment with male mice. Statistical analysis of data obtained during measurement of open field activity of male wild-type, *Qtrt1*^{+/*Gt*}, and *Qtrt1*^{*Gt/Gt*} mice (see Figure 5.4).

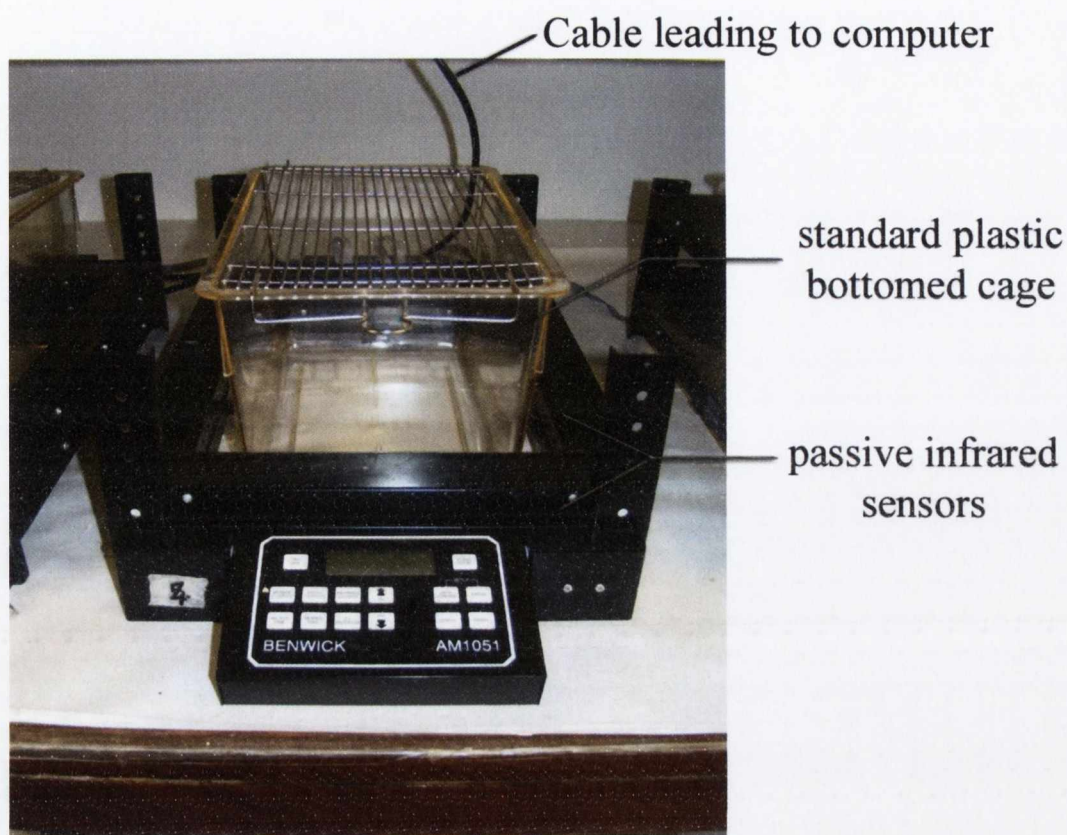


Figure 5.5 Photograph showing the experimental set-up for home cage activity measurements. Animals were placed singularly in a standard plastic bottomed cage without bedding. Behaviour was then automatically measured in the home cage activity monitor with passive infrared sensor mounted above the floor of the test cage in combination with manual scoring of stereotypy for the duration of 1 hour every 5 minutes. The data was recorded automatically on a computer.

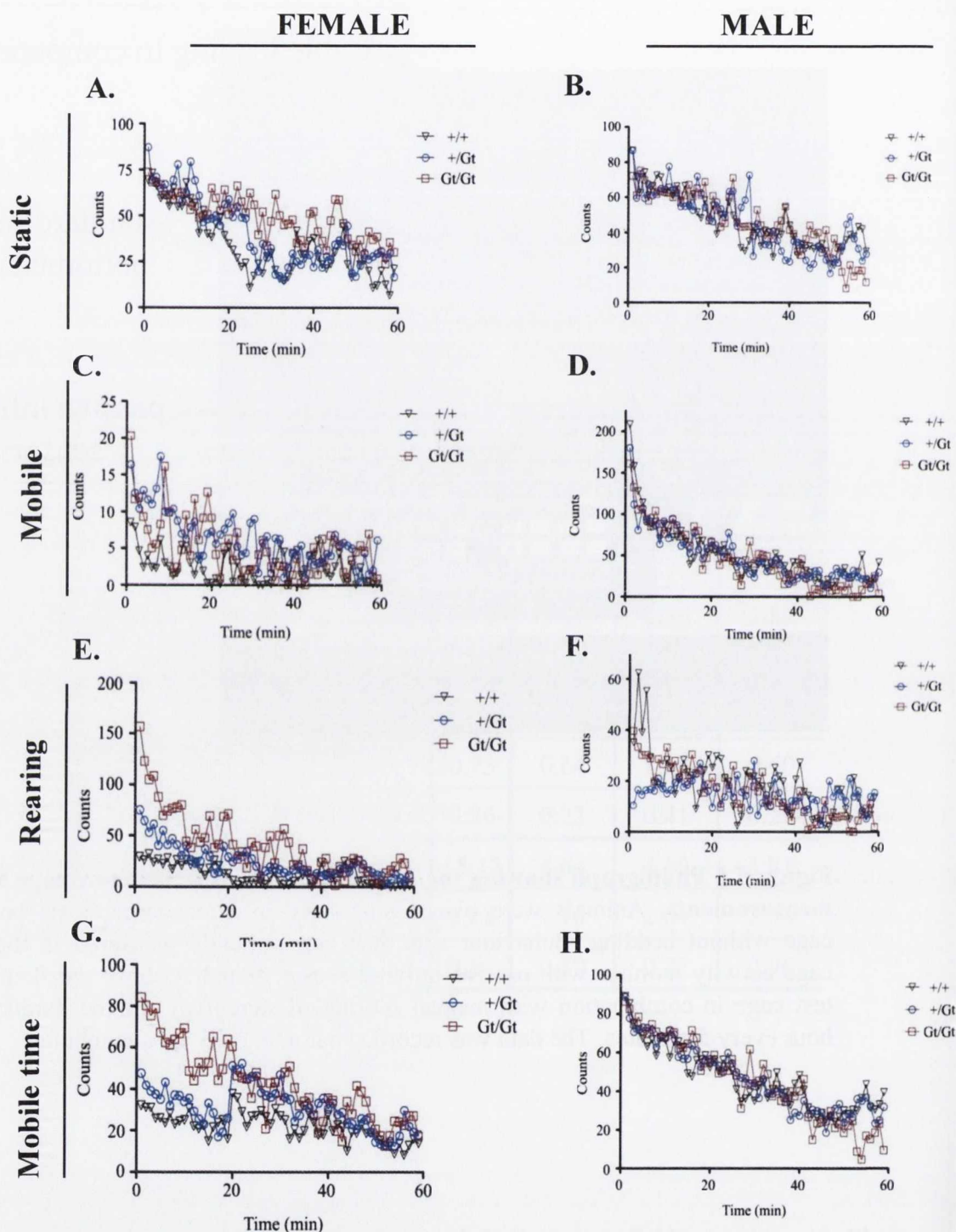


Figure 5.6 Averaged data showing home cage behaviour of wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Behavioural evaluation of home cage activity was monitored using passive infrared sensors mounted above the floor of the test cage. The behavioural patterns of static, mobile, rearing and mobile times were counted for 8 individual males and 8 individual females for wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice. The graphs represent the average behaviour of all mice within a group.

		Female			Male		
		+/+	+/Gt	Gt/Gt	+/+	+/Gt	Gt
Static	Mean	1821.00	2396.00	3000.00	2742.00	2730.00	2739.00
	SD	313.80	893.20	798.10	367.00	309.10	502.00
	SEM	111.00	315.80	282.20	129.80	109.30	177.50
Mobile	Mean	354.90	1415.00	2502.00	3077.00	2929.00	2722.00
	SD	133.70	1582.00	704.70	1083.00	1451.00	973.10
	SEM	47.26	559.30	249.10	382.70	513.10	344.00
Rearings	Mean	106.00	377.50	827.90	1080.00	877.00	1043.00
	SD	185.60	448.90	455.60	919.80	1046.00	818.20
	SEM	65.60	158.70	161.10	325.20	369.70	289.30
Mobile Time	Mean	1156.00	1876.00	2566.00	2758.00	2765.00	2771.00
	SD	130.00	1033.00	482.20	848.50	846.40	676.70
	SEM	45.95	365.10	170.50	300.00	299.20	239.30
Grooming	Mean	104.50	239.00	222.00	251.50	165.50	243.50
	SD	55.85	81.17	11.78	58.01	26.44	48.67
	SEM	27.92	40.59	5.89	29.01	13.22	24.34

Table 5.3. Analysis of data obtained during home cage activity experiment with female and male mice. Statistical analysis of data obtained during measurement of home cage activity of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 5.7).

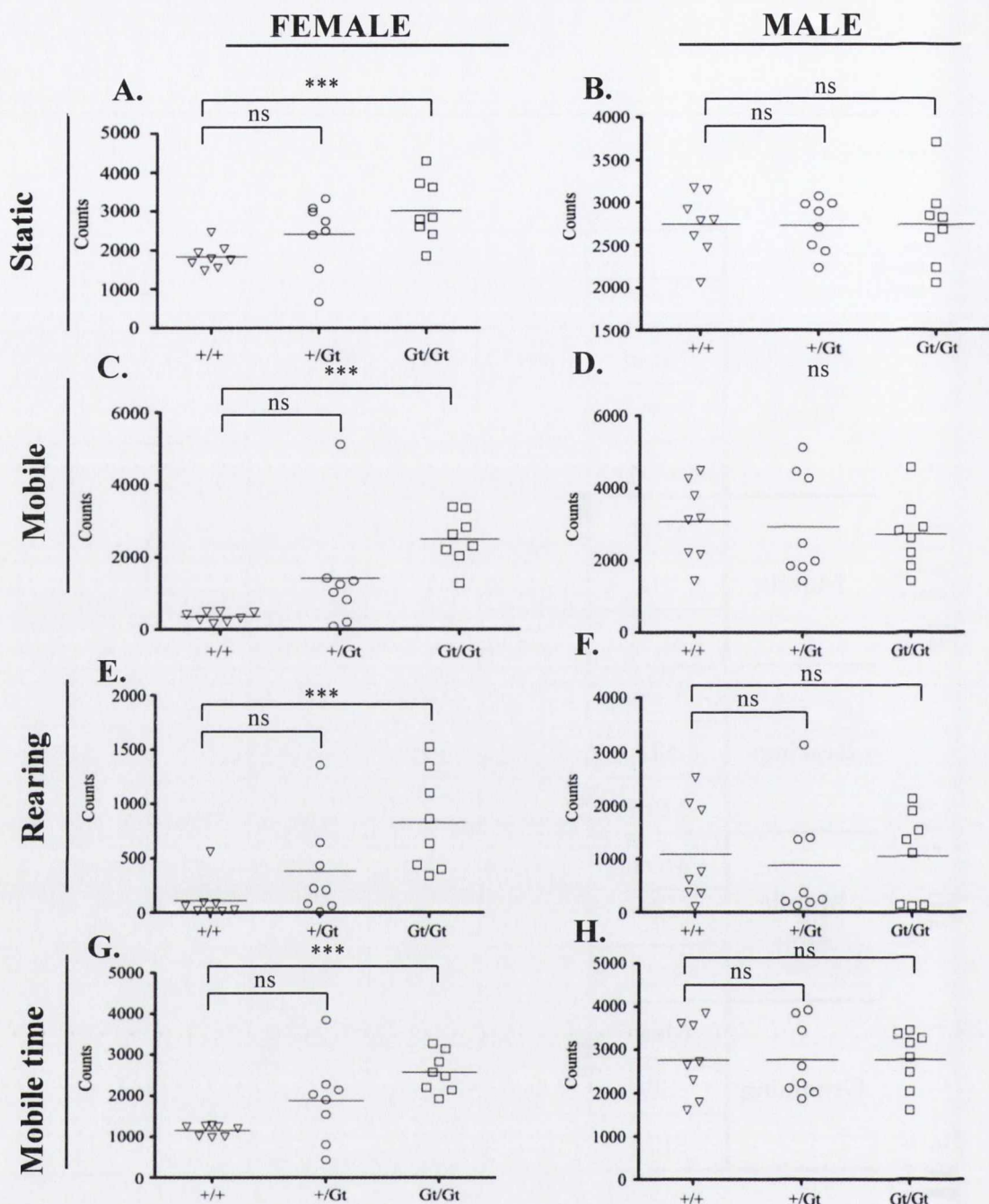


Figure 5.7 Graph representation of home cage behaviour of wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Behavioural evaluation of home cage activity was monitored using passive infrared sensors mounted above the floor of the test cage. The behavioural patterns of static, mobile, rearing and mobile times were counted for 8 individual males and 8 individual females for wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice. The graphs show the behavioural phenotype of each mice. Statistical analysis was carried out using the Student t-test (*P<0.05, **P<0.01, ***P<0.001). (See Table 5.3 for Mean values, SD and SEM)

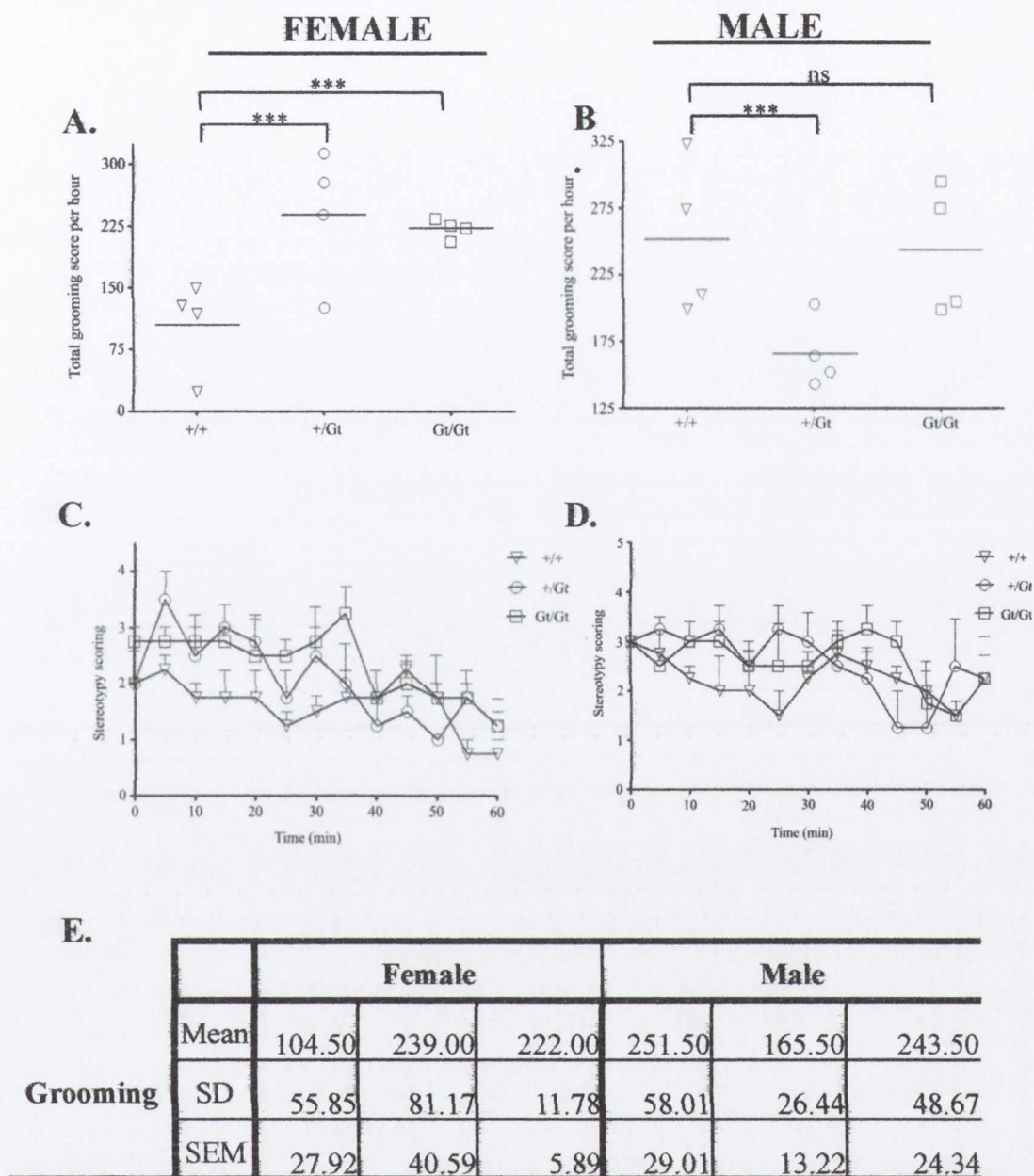


Figure 5.8 Measurement of Stereotypy behaviour in wild type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Total duration of grooming per hour in female (A) and male (B) wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) mice during the home cage activity experiment. Stereotypy scale rating scores per hour for female (C) and male (D) wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) mice by time sampling method with 5 minute interval recording. (E) Statistical analysis of data obtained during measurement of total duration of grooming per hour in female and male mice (see Figure 5.8 A&B).

5.3 Discussion

For the purposes of this study it was decided to look more closely at the effect of queuine on behaviour. *Qtrt1^{Gt/Gt}* mice have no ability to synthesize queuosine-tRNA (study carried out in our laboratory by Coilin Boland) and it was shown that they have impaired tyrosine production and differences in pteridine levels when compared to wild-type. As was mentioned before impaired tyrosine production and accumulation of phenylalanine can result in PKU disease which if left untreated can lead to the development of mental retardation and other neurological symptoms [75-78]. Deficiency in any of the components involved in BH₄ biosynthesis and regeneration also can cause several neurological diseases such as Parkinson's disease [166] and Alzheimer's disease [167]. Work carried out by Marks and Farkas [64] also showed that in the absence of tyrosine and queuine mice produce several symptoms suggestive of neurological abnormalities.

On the basis of observations obtained in this study, it was decided to examine the location of TGT and QTRTD1 in the brain. The results showed that both proteins are expressed in many regions throughout the brain with TGT being predominantly expressed in cerebrum and limbic region and QTRTD1 was found to be high in posterior cortex.

It was then decided to look at the behavioural traits associated with the absence of queuosine-tRNA. Behavioural differences were observed in both home cage and open field environments. Placing a mouse in an open field can produce a moderate level of anxiety due to a lot of open space without possibility of escape [189]. The initial response to a novel environment can be low level of activity or even freezing for a short period of time due to anxiety or perceived danger [180, 182]. After that period of time, mice generally are more active, covering larger areas of the open field which are descriptive factors of general activity and/or exploration levels [190].

Results obtained from open field testing showed that the initial response to the novel environment is comparable in all three genotypes. However, the difference can be seen in female *Qtrt1^{Gt/Gt}* mice after the initial 10 minutes acclimatisation period. *Qtrt1^{Gt/Gt}* female mice spent more time in inner zone than outer zone showing lower levels of anxiety and greater level of exploration when compared to wild-type mice and *Qtrt1^{+/-Gt}* female mice. Interestingly male mice showed no differences in the time spent in inner and outer zones.

Home cage activity testing allowed observation of wild-type and TGT genetrapped mice without contribution of any external factors. The results obtained showed that female *Qtrt1*^{Gt/Gt} mice are more active in the initial half an hour of the experiment when compared to wild-type. They spent more time moving around the cage, sniffing the walls and the floor and had higher frequency of rearing. In the case of the wild-type mice their locomotion was irregular and the frequency of rearing was low. It was also found that in female *Qtrt1*^{+ /Gt} and *Qtrt1*^{Gt/Gt} mice the duration of the total time spent rearing per hour is higher than wild-type mice. Overall these results might indicate that TGT genetrapped mice are less anxious and are more ready to explore their environment. The increase in rearing has been previously used as a measure of orienting behaviour [191] and the duration of rearing was also used as a measure of non-selective attention [192]. So the increase in mobility and rearing could mean that *Qtrt1*^{Gt/Gt} mice exhibit a more hyperactive and exploratory behaviour. Again in the course of home cage activity testing male mice displayed no differences.

The measurement of stereotypy behaviour showed higher stereotypy in both female and male *Qtrt1*^{+ /Gt} and *Qtrt1*^{Gt/Gt} mice when compared to wild-type mice. It was interesting that TGT genetrapped male mice exhibited different stereotypic behaviour to wild-type mice since automatic scoring did not show any significant difference in male behaviour.

In summary, the absence of queuosine-tRNA in *Qtrt1*^{Gt/Gt} mice results in behavioural changes but only in females. Although male TGT genetrapped mice displayed differences in stereotypy behaviour the automatic detection of difference behaviour characteristics did not show significant change. However, female *Qtrt1*^{Gt/Gt} mice exhibit higher general activity and exploration levels. This may coincide with the behavioural hyperactivity characteristic that is found in PKU patients. The increase and duration of rearing might also show the increase in orientating behaviour and non-selective attention. Further experiments will need to be conducted to understand the behavioural abnormalities of queuosine deficient animals more clearly and explore how they relate to the altered behaviour of PKU patients.

Chapter 6

Neurological and biochemical comparison of wild-type and TGT genetrap mice

6.1 Introduction

The research of Marks and Farkas [64] on germ-free mice, provided evidence that a link exists between queuine and the synthesis of the amino acid tyrosine. They showed that the removal of tyrosine and queuine from the diet of germ-free mice resulted in squinting, convulsion, laboured breathing and death, among other symptoms. We consider it highly unlikely that queuine deficiency has an impact on downstream functions of tyrosine, since re-administration of tyrosine to queuine deficient mice precluded the development of any symptoms. And our results concur with this conclusion since we observed the accumulation of the BH₄ metabolite BH₂. Potentially, TGT genetrap animals may experience decreased amounts of tyrosine and BH₄.

As mentioned before, tyrosine has an important role in the brain as it can be further hydrolysed to L-DOPA by the tyrosine hydroxylase enzyme. L-DOPA formation is a rate limiting step in the biosynthesis of dopamine, norepinephrine (or noradrenaline) and epinephrine (or adrenaline), which are known as catecholamines. The production of catecholamines is localised to specific neuronal tracts in the brain, chromaffin cells, sympathetic nerves and sympathetic ganglia [193]. Maintaining a healthy level of catecholamine neurotransmitter is crucial for normal neurological development. Genetic disorders that disrupt catecholamine biosynthesis gives rise to severe neurological defects and in some circumstances death [194]. Studies carried out with knockout mice showed that an absence of the tyrosine hydroxylase gene results in approximately 90% lethality *in utero* [179]. The biosynthesis of dopamine plays an important role in the lasting internal coding of motor skills. It is believed to be important in teaching the brain to acquire new behaviour. Noradrenaline is involved in the control of seizures such as those found in epilepsy, but it is less significant when compared to other biogenic amines in this role and is primarily involved in the control of blood circulation [195]. Importantly, BH₄ is also a necessary cofactor for the synthesis of two other major classes of neurotransmitter, serotonin and nitric oxide.

Serotonin, also known as 5-hydroxytryptamine, is a member of indoleamine family of organic compounds such as the hormone melatonin and hallucinogen N, N-dimethyltryptamine. It is formed through hydroxylation by the tryptophan hydroxylase enzyme followed by decarboxylation by aromatic L-amino acid decarboxylase. Serotonin is primarily found in the gastrointestinal tract, platelets, and in the central nervous system [196] and is thought to be involved in the regulation of the majority of brain functions. Disruption of the serotonergic system has been shown to result in neurological pathogenesis and psychiatric disorders [197-198]. Serotonin neurons extend to numerous locations in the brain such as the limbic and cortical regions, the midbrain and the hindbrain. In fact, serotonin receptors of various subtypes, can be found in all major brain regions and some individual neurons have an ability to express several serotonin receptors [199]. It is this abundance of serotonin and serotonin receptors that results in its ability to modulate so many behavioural and neuropsychological processes including mood, anger, aggression, appetite, memory, sexuality, perception and many others [200-202].

However, the importance of serotonin is not confined solely to the central nervous system. It was shown that serotonin has endocrine and gastrointestinal functions [203-206], it has a role in regulation of energy balance and food intake [207], and it has functions in cardiovascular and pulmonary [208-209] physiology [210-211]. Serotonin has been shown to play an important role in control of vascular resistance, blood pressure, hemostasis and platelet function. In blood vessels serotonin can either cause vasoconstriction or vasodilation depending on the type of serotonin receptors expressed in the vessel wall and surrounding smooth muscle tissue [212]. Platelets have no serotonin synthesizing enzymes and therefore obtain their supply of serotonin by uptake from the plasma by serotonin transporter. Once platelets are activated, serotonin is secreted by platelet dense granules and this in turn results in platelet aggregation and vasoconstriction of surrounding blood vessels which can result in hemostasis [213]. Studies carried out in serotonin deficient mice suggested that serotonin might have a role in setting of metabolic rate and temperature control [214]. When placed in cold environment these mice displayed a very rapid hypothermic response.

The BH₄ is also a cofactor for the major signalling molecule nitric oxide, which is a small free radical gaseous molecule. The first study on nitric oxide function was

published in 1980 by Furchgott *et al.* [215] where it was given a name of endothelium-derived relaxing factor because of its location and function on smooth muscle in blood vessel wall. Later Palmer *et al.* [216-217] demonstrated the endothelium-derived relaxing factor was in fact nitric oxide. Nitric oxide is important in many biological functions. In addition to its role as a relaxant of smooth muscle nitric oxide is a key signalling molecule involved in homeostasis of the gastrointestinal tract [218]. In the skin it is thought to be involved in maintenance of barrier function and determination of blood flow in microvasculature [219]. It was also shown that nitric oxide produced by macrophages and hepatocytes treated with cytokines may have a function in host responses to infectious pathogens [197, 220]. As well as this nitric oxide was suggested to have a role in learning and memory possibly due to physiological role of NO in synaptic plasticity [198, 221] and in motor control [222-223]. Nitric oxide synthesis is carried by an enzyme known as nitric oxide synthase (NOS). NOS can be found in almost all cells and tissues and can exist as three different isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The synthesis of nitric oxide from L-arginine with the subsequent formation of L-citrulline is carried out in NADPH dependent manner by NOS which requires Ca^{2+} /calmodulin, FAD, FMN, and BH_4 as the cofactors [224-227].

It has been suggested that under certain conditions NOS enzyme can produce superoxide [228-231]. The production of superoxide by eNOS is primarily regulated by the optimum concentration of BH_4 and occurs due to 'uncoupling of NOS' [232-233]. The accumulation of superoxide in endothelium had been suggested to have a role in hypercholesterolemia, atherosclerosis, and hypertension. Of particular relevance to this study, the ratio of BH_4 to BH_2 is a major factor in eNOS uncoupling [119] [121]. The administration of BH_4 improves endothelial function by promoting the proper coupling of the NOS reaction thereby preventing the formation of reactive oxygen species [234-237].

The degradation of catecholamine and indoleamine neurotransmitters occurs through several alternative pathways which are carried out by least three different intracellular enzymes, most notably MAO. MAO is involved in inactivating catecholamines through O-methylation and deamination. MAO deficiency is a cause for several inherited disorders such as Norrie disease which results in blindness, deafness, and variable mental retardation [238].

From the work carried out by Patti Hayes in our laboratory it was found that queuine deficient cells in culture show bioenergetic changes resulting in increased glycolysis and glutaminolysis with concomitant upregulation of lactate and ammonia production (unpublished observations). Energy is especially important for proper brain and body function. Deficiency in brain energy levels has been proposed to be one of the causes of behavioural problems in Attention-deficit/hyperactivity disorder (ADHD). These behavioural problems are thought to arise due to impairment of astrocyte uptake of glucose from blood capillaries, its utilization by conversion to lactate and storage as glycogen [239].

Given the results obtained from Patti's work it was decided that a biochemical approach may prove effective in defining the energetic changes in TGT genetrapped mice. NMR spectroscopy is becoming a key tool for studying metabolic processes in living systems. It is a useful technique for studying composition of biofluids, cells and tissues and for providing crucial information on gene function [240]. The spectrum of biofluid or tissue obtained by ^1H NMR consists of thousands of well-resolved signals. The intensity of these signals correlate to the concentration of these metabolites in the sample and provides us with a fingerprint of an organism being studied [241]. By comparing proton-NMR (^1H -NMR) spectra of biofluids from wild-type and TGT genetrapped mice it is hoped that differences may be discernable between these animals on a small molecule level.

6.2 Results

6.2.1 Biogenic Amines in wild-type and *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt/Gt*}

Since tyrosine biosynthesis is compromised after exogenous phenylalanine administration over a period of time and pteridine levels were found to be altered in heterozygous and homozygous TGT genetrapped mice, it was decided to compare the levels of biogenic amines in all three genotypes.

Biogenic amines were measured in female and male mice (n=4 per genotype for each gender). The brain was divided into six fine sections: cerebellum, frontal cortex, hypothalamus, striatum, hippocampus and the rest of the brain. Each of these parts was then analysed for the presence of biogenic amines. The separation of biogenic amines was carried out on an LI Chrosorb RP-18 column and the retention times carried between 5 to 25 minutes.

In the cerebellum section of female mice neurotransmitter levels seemed mostly unchanged between genotypes (Figure 6.1.1 A, (See Table 6.1A for Mean values, SD and SEM)). The differences in L-DOPA, DA, NA, HVA and 5HT were not significant. However, the level of DOPAC, which is a metabolite of L-DOPA and dopamine, seemed to be significantly higher and the level of 5HIAA, serotonin metabolite, was found to be significantly lower in *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt/Gt*} mice when compared to wild-type. The level of DOPAC increased from ~ 50ng to 90ng of brain tissue and the level of 5HIAA was found to decrease from ~290ng to 210 per gram of brain tissue. As well as that the ratio of 5HT to 5HIAA conversion showed a slight decrease in *Qtrt1*^{*Gt/Gt*} mice (Table 6.1 A.), DA to DOPAC conversion ratio showed more than a 50% increase in *Qtrt1*^{+/*Gt*} and *Qtrt1*^{*Gt/Gt*} mice (Table 6.1 C), a significant increase was also observed in DA to HVA ratio and the DOPAC+HVA/DA ratio (Table 6.2 A and C, respectively).

In cerebellum of male mice the changes in NA and DOPAC were found to be non-significant (Figure 6.1.1 B, (See Table 6.1B for Mean values, SD and SEM)). The levels of the rest of the biogenic amines measured were found to be significantly changed. The level of L-DOPA increased from ~28ng to 50ng per gram of brain tissue, the level of DA decreased from ~14ng to ~4ng per gram of brain tissues, the values for HVA increased from ~85ng to 180ng per gram of brain tissue and the levels of 5HT and 5HIAA were

found to decrease from 350ng to 250ng and from 180ng to 140ng per gram of brain tissue, respectively. The dopamine turnover to DOPAC was found to be significantly lower in *Qtrt1*^{+/-Gt} and *Qtrt1*^{Gt/Gt} mice when compared to wild-type (Table 6.1 D).

In the frontal cortex region of female mice no difference was observed in the levels of L-DOPA, DA, NA and DOPAC (Figure 6.1.1 C, (See Table 6.2A for Mean values, SD and SEM)). The levels of HVA, 5HT and 5HIAA were found to be decreased in *Qtrt1*^{Gt/Gt} mice from ~800ng to ~600ng, from ~760ng to ~660ng and from ~295ng to 245ng, respectively, per gram of brain tissue. The ratio of dopamine to HVA conversion in TGT genetrapped mice was also found to be lower when compared to wild-type (Table 6.2 A).

In the frontal cortex region of male mice no difference was observed in the levels of L-DOPA, DA, NA, HVA and 5HIAA (Figure 6.1.1 D, (See Table 6.2B for Mean values, SD and SEM)). The levels of DOPAC and 5HT increased in *Qtrt1*^{Gt/Gt} mice from ~55ng to ~50ng and ~360ng to ~270ng, respectively, per gram of brain tissue when compared to wild-type. The ratio of dopamine conversion to HVA was found to be lower in *Qtrt1*^{Gt/Gt} mice and the ratio DOPAC+HVA/DA was found to be higher (Table 6.8 B and D).

In the hypothalamic region of female brain no significant difference was observed in L-DOPA levels (Figure 6.1.1 E, (See Table 6.3A for Mean values, SD and SEM)). The values for the rest of biogenic amine neurotransmitters were found to be lower. The level of DA decreased from ~710ng to 670ng and the level of NA decreased from ~3900ng to 3200ng per gram of brain tissue. The value for DOPAC decreased from ~270ng to ~180ng and HVA from ~790ng to 670ng per gram of brain tissue. The values for 5HT and its metabolite 5HIAA decreased from ~2600ng to 2100ng and ~1300ng to ~1000ng, respectively, per gram of brain tissue. The ratios of DA to DOPAC (Table 6.1 C), DA to HVA (Table 6.8 A) and the ratio of DOPAC+HVA/DA (Table 6.8 C) were found to be significantly lower in *Qtrt1*^{Gt/Gt} mice when compared to wild-type.

In hypothalamus region of male brains no significant changes were observed in L-DOPA, DA, NA, DOPAC, HVA and 5HIAA (Figure 6.1.1 F, (See Table 6.3B for Mean values, SD and SEM)). The value for 5HT for *Qtrt1*^{Gt/Gt} mice increased from ~1900ng to ~2200ng per gram of brain tissue. The ratios of 5HT to 5HIAA (Table 6.7 B) and DA to HVA (Table 6.8 B) conversions were found to be decreased in *Qtrt1*^{Gt/Gt} mice when compared to wild-type and the ratios of DA to DOPAC (Table 6.7 D) and

DOPAC+HVA/DA (Table 6.8 D) were found to be increased in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

In the striatum region of female brains analysed no significant difference was observed in the levels of NA, DOPAC and 5HT (Figure 6.1.2 A, (See Table 6.4A for Mean values, SD and SEM)). The levels of L-DOPA and NA were decreased from ~260ng to 170ng and 630ng to 550ng, respectively per gram of brain tissue and the values for HVA and 5HIAA were found to decrease from ~4450ng to 2900ng and ~860ng to ~580ng per gram of brain tissue. The ratio of 5HT to 5HIAA (Table 6.7 A) conversion as well as DOPAC+HVA/DA (Table 6.8 D) were found to be lower in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

In striatum section of male brains no difference was observed in the levels of L-DOPA, NA, DOPAC and 5HT (Figure 6.1.2 B, (See Table 6.4B for Mean values, SD and SEM)). The levels of DA, HVA and 5HIAA increased from ~11,480ng to ~17,000ng, ~2900ng to ~3600ng and ~490ng to ~640ng, respectively, per gram of brain tissue. The ratios of 5HT to 5HIAA (Table 6.7 B) conversion and DOPAC+HVA/DA (Table 6.8 D) were found to be higher in TGT genetrapp mice when compared to wild-type and the ratios of DA to DOPAC (Table 6.7 D) and DA to HVA (Table 6.8 B) conversion were found to decrease in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

In the hippocampus region of female brains significant decreases were observed in DA and DOPAC with value falling from ~180ng to ~60ng and ~83ng to ~60ng, respectively, per gram of brain tissue (Figure 6.1.2 C, (See Table 6.5A for Mean values, SD and SEM)). The rest of the regions had comparable amounts of biogenic amines. The ratio of DA to DOPAC (Table 6.7 C) conversion and DOPAC+HVA/DA (Table 6.8 C) ratio were found to be lower in *Qtrt1^{Gt/Gt}* mice and the ratio of DA to HVA (Table 6.8 A) conversion was found to be higher in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

In male mice almost no changes of the amount of biogenic amines present in hippocampus region of the brain were observed (Figure 6.1.2 D, (See Table 6.5B for Mean values, SD and SEM)). Only 5HIAA level was found to be significantly lower in *Qtrt1^{Gt/Gt}* mice decreasing from ~490ng to ~340ng per gram of brain tissue. However, the ratios of 5HT to 5HIAA (Table 6.7 B), DA to DOPAC (Table 6.7 D), DA to HVA (Table 6.8 B) conversions as well as DOPAC+HVA/DA (Table 6.8 D) ratio were all found to be significantly lower in *Qtrt1^{Gt/Gt}* mice when compared to wild type.

In the rest of the female brain, which included sections that were not included in fine sections, only the level of L-DOPA was found to be unchanged (Figure 6.1.2 E, (See Table 6.6A for Mean values, SD and SEM)). The levels of DA, NA and DOPAC fell from ~630ng to ~498ng, ~468ng to ~320ng and ~141ng to ~135ng, respectively per gram of brain tissue. Levels of HVA, 5HT and 5HIAA were also found to decrease from ~395ng to ~320ng, ~470ng to ~396ng and ~296ng to ~215ng, respectively, per gram of brain tissue. The conversion ratio of 5HT to 5HIAA (Table 6.7 A) was also found to decrease but an increase was observed in the rate of DA to DOPAC (Table 6.7 C) conversion in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

In male brain analysis of the rest of the brain sample showed increase in DA from ~460ng to ~480ng and decrease in HVA from ~330ng to ~290ng per gram of brain tissue in *Qtrt1^{Gt/Gt}* mice when compared to wild-type (Figure 6.1.2 F, (See Table 6.6B for Mean values, SD and SEM)). No differences were found in other levels of biogenic amines. The ratio of DA to HVA (Table 6.8 B) conversion was found to be lower and the DOPAC+HVA/DA (Table 6.8 D) ratio was found to be higher in *Qtrt1^{Gt/Gt}* mice when compared to wild-type.

This difference in the levels of biogenic amines in wild-type and TGT gene-trap mice must mean that queuine has a significant role not only in maintaining tyrosine biosynthesis and proper levels of pterins but also in maintaining healthy levels of biogenic amines.

6.2.2 Comparison of biogenic amine levels in wild-type with values from literature

In 2005 a paper was published by Van Dam *et al.* where the levels of neurotransmitter and related compounds in C57Bl/6 mice were published [242]. These values were used in this part of the study to compare with those obtained in our wild-type control mice. It was decided to make a table comparing approximate values for our female and male wild-type and C57Bl/6 mice of unknown gender analysed by Van Dam (See Table.6.9).

Firstly, values for biogenic amines were compared between female wild-type mice and C57Bl/6 used by Van Dam (Table 6.9 A). It was found that the values for NA across different brain regions were approximately the same with the exception of hypothalamus

where it was found to be higher. Dopamine values were also comparable except in hippocampus it was found to be lower. DOPAC was found to be lower in all of the brain regions analysed. HVA was found to be lower in cerebellum and hippocampus but higher in striatum. Serotonin (5HT) was found to be lower in cerebellum but higher in hypothalamus and 5HIAA was lower in the frontal cortex and higher in the hypothalamus.

A comparison of biogenic amines levels in male wild-type mice and mice used by Van Dame also showed a lot of differences (table 6.9 B). All biogenic amine levels were found to be lower in all of the brain regions compared, the only exception being 5HT in the hypothalamus, which was found to be higher in our male wild-type mice.

6.2.3 ^1H NMR analysis of plasma, liver and brain samples of wild-type, $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ mice

^1H NMR analysis of plasma, liver and brain of wild-type and TGT genetrapped mice was carried out to determine if any small molecule differences (i.e. lipids, carbohydrates, amino acids and metabolites) exists between these genotypes.

Combined representative ^1H NMR spectra of plasma from wild-type, $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ mice are shown in Figure 6.2. The spectra show resonances from low molecular weight metabolites, with contributions from high molecular weight lipoproteins. The differences in spectra are highlighted and have been magnified. The identity of the resonances assigned in the spectra (numbered) has been based on literature queries [243], including those resonances from lipids, glucose, 3-D-hydroxybutyrate, acetate, alanine, glutamine, pyruvate, and lactate (Table 6.10). It was also possible to calculate the amounts of lactate, alanine, creatine, 3-D-hydroxybutyrate and choline by normalising the values obtained to an internal TSP standard (Figure 6.3). Results showed a marked decrease ($p < 0.001$) in the levels of lactate (Figure 6.3 A and B), alanine (Figure 6.3 C) and creatine (Figure 6.3 D) for $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ mice compared to wild-type mice. There was no significant difference in values obtained for 3-D-hydroxybutyrate (Figure 6.3 E) or choline (Figure 6.3 F) (See Table 6.11 for Mean values, SD and SEM).

To characterize the biochemical status of the brain from wild-type and $Qtrt1^{+/Gt}$ and $Qtrt1^{Gt/Gt}$ mice, ^1H NMR spectra were analysed for both lipid and aqueous soluble fractions of the tissue. The representative spectra are given in Figures 6.4 and 6.5,

respectively. The differences in spectra have been highlighted and magnified. The resonances were then assigned in those magnified regions (Tables 6.12 and 6.13, respectively), through comparison to peak positions designated in the literature [244].

The analysis of the lipid soluble brain extract spectra (Figure 6.4) enabled assignment of metabolites such as cholesterol and unsaturated fatty acyl moieties (Table 6.12). All of the samples were normalised to TSP standard and therefore a relative comparison of metabolite levels was possible. A lot of differences were found in the range δ (^1H shift) 1.5-3.0 ppm as well as δ 3.4-4.6 ppm. Differences in the levels were also observed in the range δ 0.62-0.72, δ 0.84-0.94 and δ 0.96-1.04 ppm. ^1H NMR spectra of aqueous soluble brain tissue extract from wild-type and *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice are given in Figure 6.5. These spectra showed only low molecular weight metabolites. All of the samples were normalised to TSP standard so comparison of the level of metabolites was possible. The differences in spectra were magnified and resonances in those magnified regions were assigned (Table 6.13), on the basis of literature values [244-245]. The analysis of aqueous soluble brain extract allowed the assignment of metabolites such as sugars and amino acids, lactate, uridine triphosphate, inosine and adenosine-diphosphate (Table 6.13).

Characterisation of biochemical composition of liver from wild-type and *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice was also carried out. ^1H NMR spectra was measured in lipid and aqueous soluble extracts of the tissue. Lipid soluble liver extract ^1H NMR spectra for wild-type and TGT genetrap mice are presented in Figure 6.6. The differences in spectra were magnified and resonances were then assigned in those magnified regions (Table 6.14) on the basis of literature values [244]. The detection of metabolites such as cholesterol and unsaturated fatty acyl moieties was possible and their levels were compared these samples to internal TSP standard (Figure 6.6). Differences in levels of metabolites were in all of the regions magnified.

^1H NMR spectra was also obtained for aqueous soluble liver extract from wild-type and *Qtrt1*^{+/Gt} and *Qtrt1*^{Gt/Gt} mice (Figure 6.7). The metabolites present in this spectrum were of low molecular weight. The differences in spectra were magnified and compared by normalising samples to internal TSP standard. The resonances in magnified regions were assigned (Table 6.15) on the basis of literature values [244-245]. The detection of

metabolites such as glucose, uridine triphosphate, inosine, adenosine-diphosphate, phenylalanine, histidine, nicotinurate and uridine was possible in those magnified regions (Figure 6.7 and Table 6.15). The levels of these metabolites were found to be different between genotypes.

In conclusion, ^1H NMR analysis of plasma and lipid and aqueous extracts from brain and liver tissue displayed a number of differences in the level of metabolites present.

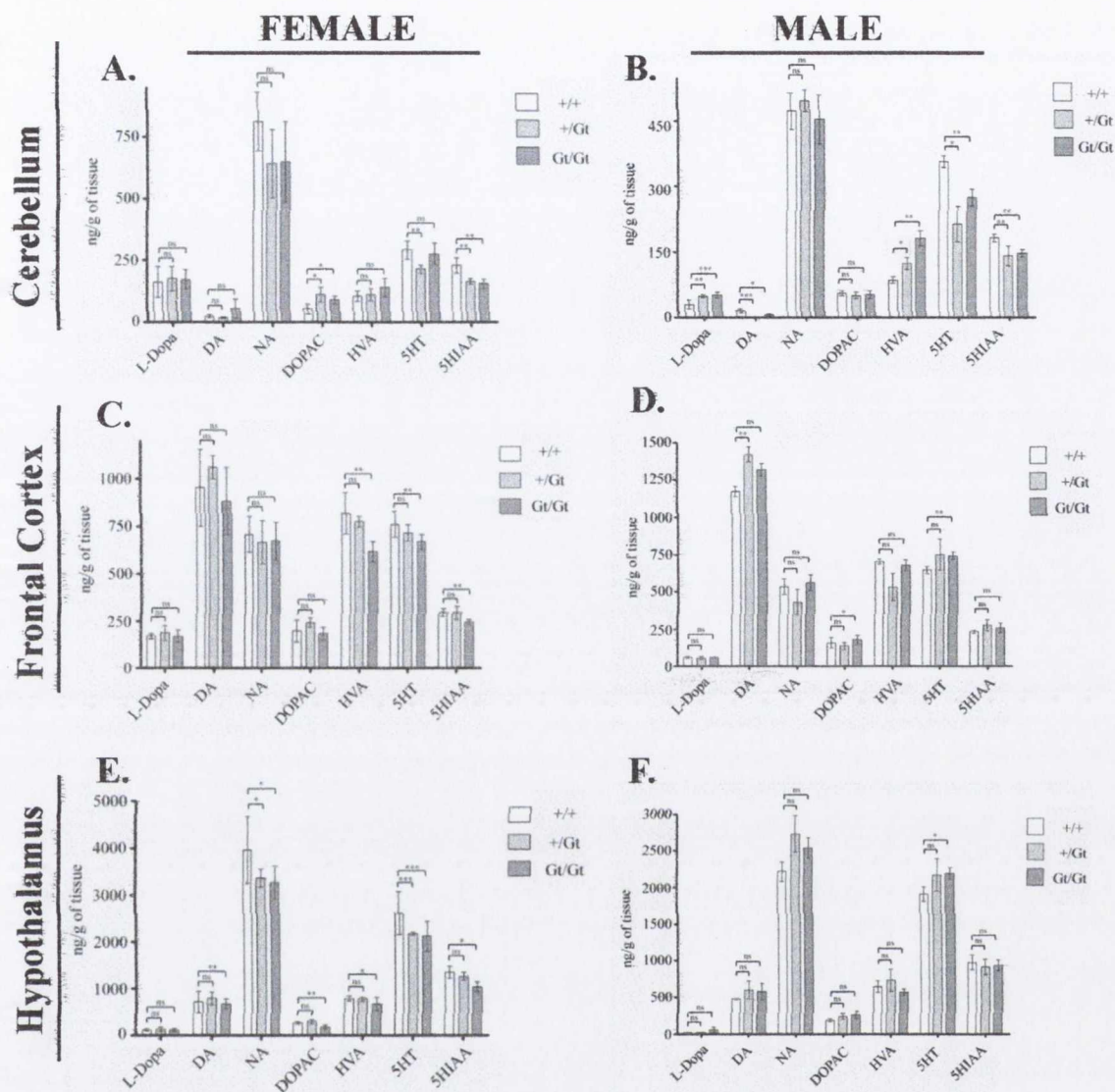


Figure 6.1.1 Biogenic Amines levels in fine brain sections of female and male TGT genetrapped mice. Analysis of biogenic amines in wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice by HPLC with electrochemical detection. The levels of noradrenaline (NA), levodopa (L-DOPA), dopamine (DA), 5-hydroxyindolacetic acid (5HIAA), homovanillic acid (HVA) and serotonin (5HT) were measured in cerebellum (A, B) (See Table 6.1 for Mean values, SD and SEM), frontal cortex (C, D) (See Table 6.2 for Mean values, SD and SEM) and Hypothalamus (E, F) (See Table 6.3 for Mean values, SD and SEM) brain regions of female and male mice. The concentration of biogenic amines is expressed as ng/g of tissue, and data represent the mean $\pm$ SEM (n=4 mice per genotype). *P<0.05, **P<0.01, ***P<0.001, Student's t-test

A.

Cerebellum		Female					
	Biogenic Amines	Mean	SEM	Mean	SEM	Mean	SEM
	L-DOPA	161.30	62.65	174.60	49.25	170.85	40.19
	DA	23.68	5.83	18.00	3.29	52.40	38.64
	NA	811.35	120.23	640.50	138.26	647.05	164.02
	DOPAC	52.63	19.82	110.53	29.92	88.23	15.80
	HVA	103.88	22.21	109.50	24.29	138.50	37.82
	5HT	291.00	37.47	213.53	16.77	275.18	43.72
	5HIAA	228.43	32.03	164.65	12.44	154.05	19.15

B.

Cerebellum		Male					
	Biogenic Amines	Mean	SEM	Mean	SEM	Mean	SEM
	L-DOPA	28.55	10.04	48.00	3.12	50.65	7.05
	DA	14.55	4.51	0.00	0.00	4.68	2.73
	NA	473.25	41.72	497.00	24.84	454.65	56.44
	DOPAC	54.98	5.54	49.30	7.16	51.53	9.73
	HVA	85.00	7.19	123.48	13.88	180.40	17.63
	5HT	356.88	13.27	213.63	40.59	274.43	19.35
	5HIAA	181.65	9.47	140.38	22.84	146.00	8.32

Table 6.1. Analysis of data obtained during biogenic amines measurement in cerebellum of female and male TGT genetrap mice. Statistical analysis of data obtained during measurement of biogenic amines in cerebellum of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.1.1 A&B).

A.

	Biogenic Amines	Female					
		Mean	SEM	Mean	SEM	Mean	SEM
Frontal Cortex	L-DOPA	170.70	12.67	186.43	39.68	169.83	33.62
	DA	952.58	204.41	1059.95	61.22	884.10	175.09
	NA	707.80	96.28	665.23	114.66	672.73	100.68
	DOPAC	197.73	59.02	242.35	25.35	185.13	33.86
	HVA	818.28	108.58	774.08	28.05	615.15	53.65
	5HT	760.08	68.32	716.23	44.13	667.93	39.25
	5HIAA	295.08	19.98	293.28	33.79	245.63	12.21

B.

	Biogenic Amines	Male					
		Mean	SEM	Mean	SEM	Mean	SEM
Frontal Cortex	L-DOPA	58.10	6.02	55.10	9.86	56.35	4.17
	DA	1172.75	33.03	1420.40	51.17	1318.05	42.80
	NA	535.98	52.72	431.08	84.82	559.93	51.42
	DOPAC	156.85	34.88	135.18	25.91	176.55	32.41
	HVA	702.45	17.40	531.68	91.58	676.60	37.73
	5HT	645.40	23.14	749.60	104.73	741.65	23.45
	5HIAA	233.58	9.46	276.78	37.53	257.38	32.23

Table 6.2. Analysis of data obtained during biogenic amines measurement in frontal cortex of female and male TGT genetrapp mice. Statistical analysis of data obtained during measurement of biogenic amines in frontal cortex of female and male wild-type, *Qtrt1*^{+/*Gt*}, and *Qtrt1*^{*Gt/Gt*} mice (see Figure 6.1.1 C&D).

A.

	Biogenic Amines	Female					
		Mean	SEM	Mean	SEM	Mean	SEM
Hypothalamus	L-DOPA	117.25	24.93	139.48	47.69	115.50	34.70
	DA	710.75	227.12	790.10	133.31	674.40	104.28
	NA	3957.85	715.08	3355.70	188.52	3257.10	348.05
	DOPAC	269.75	21.47	302.45	37.75	178.58	48.00
	HVA	788.13	60.45	775.40	42.02	673.30	145.81
	5HT	2616.18	464.72	2171.97	34.54	2119.30	322.69
	5HIAA	1346.70	130.62	1255.95	87.35	1037.20	105.24

B.

	Biogenic Amines	Male					
		Mean	SEM	Mean	SEM	Mean	SEM
Hypothalamus	L-DOPA	58.10	6.02	55.10	9.86	56.35	4.17
	DA	1172.75	33.03	1420.40	51.17	1318.05	42.80
	NA	535.98	52.72	431.08	84.82	559.93	51.42
	DOPAC	156.85	34.88	135.18	25.91	176.55	32.41
	HVA	702.45	17.40	531.68	91.58	676.60	37.73
	5HT	645.40	23.14	749.60	104.73	741.65	23.45
	5HIAA	233.58	9.46	276.78	37.53	257.38	32.23

Table 6.3. Analysis of data obtained during biogenic amines measurement in hypothalamus of female and male TGT genetrapp mice. Statistical analysis of data obtained during measurement of biogenic amines in hypothalamus of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.1.1 E&F).

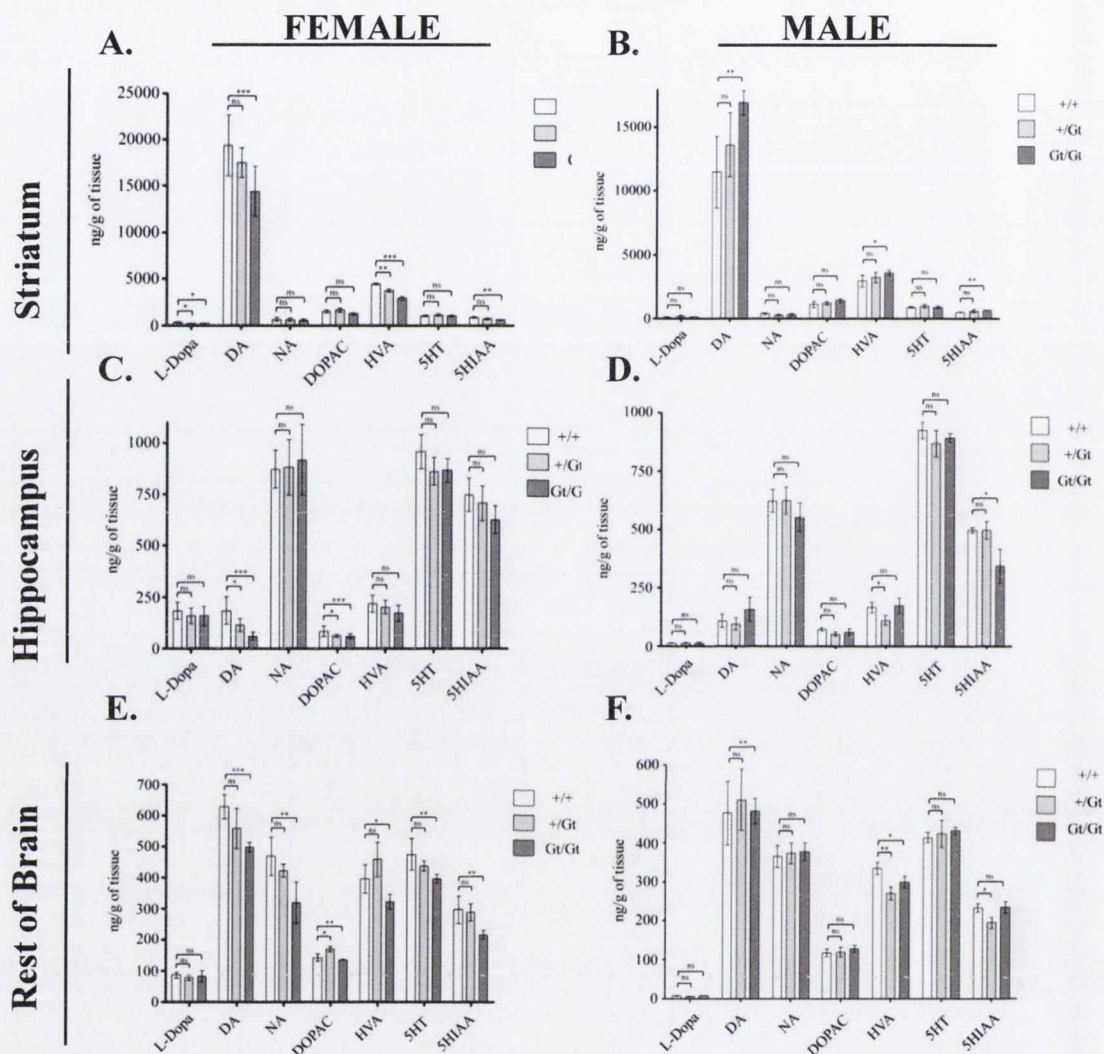


Figure 6.1.2 Biogenic Amines levels in fine brain sections of female and male TGT genetrap mice. Analysis of biogenic amines in wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice by HPLC with electrochemical detection. The levels of noradrenaline (NA), levodopa (L-DOPA), dopamine (DA), 5-hydroxyindolacetic acid (5HIAA), homovanillic acid (HVA) and serotonin (5HT) were measured in Striatum (**A, B**) (See Table 6.4 for Mean values, SD and SEM), Hippocampus (**C, D**) (See Table 6.5 for Mean values, SD and SEM) and Rest of Brain (**E, F**) (See Table 6.6 for Mean values, SD and SEM) brain regions of female and male mice. The concentration of biogenic amines is expressed as ng/g of tissue, and data represent the mean $\pm$ SEM ($n=4$ mice per genotype). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Student's t-test

A.

	Biogenic Amines	Female					
		Mean	SEM	Mean	SEM	Mean	SEM
Striatum	L-DOPA	266.08	86.38	188.53	30.43	176.60	28.82
	DA	19350.43	3298.80	17493.65	1608.47	14410.95	2710.75
	NA	630.53	192.78	593.35	192.63	554.30	142.04
	DOPAC	1466.18	161.28	1629.00	192.10	1207.18	140.56
	HVA	4450.20	74.20	3732.78	186.02	2890.33	224.01
	5HT	996.73	114.05	1087.95	123.07	990.40	59.67
	5HIAA	858.85	86.80	709.40	85.81	576.08	64.51

B.

	Biogenic Amines	Male					
		Mean	SEM	Mean	SEM	Mean	SEM
Striatum	L-DOPA	78.27	37.62	159.23	96.78	99.18	26.40
	DA	11477.60	2805.32	13608.35	2522.62	16926.02	971.95
	NA	363.18	51.93	268.93	64.44	306.00	94.97
	DOPAC	1082.03	261.21	1194.08	132.96	1396.20	157.93
	HVA	2943.40	473.81	3216.95	399.74	3569.93	219.73
	5HT	866.95	35.13	975.00	148.88	876.13	70.48
	5HIAA	494.78	38.92	582.70	102.45	636.58	34.06

Table 6.4. Analysis of data obtained during biogenic amines measurement in striatum of female and male TGT genetrap mice. Statistical analysis of data obtained during measurement of biogenic amines in striatum of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.1.2 A&B).

A.		Female					
Hippocampus	Biogenic Amines	Mean	SEM	Mean	SEM	Mean	SEM
	L-DOPA	183.53	41.18	158.30	38.23	157.83	47.34
	DA	183.78	67.36	113.95	31.99	59.28	18.79
	NA	872.40	92.80	881.28	136.14	917.73	169.90
	DOPAC	83.20	25.76	60.63	6.25	59.90	13.08
	HVA	218.25	41.33	201.40	33.76	170.83	39.74
	5HT	958.23	82.37	861.48	67.56	866.40	56.74
	5HIAA	747.98	80.44	707.13	84.84	626.98	66.54

B.		Male					
Hippocampus	Biogenic Amines	Mean	SEM	Mean	SEM	Mean	SEM
	L-DOPA	12.20	1.46	11.93	1.76	12.25	3.29
	DA	106.45	28.82	94.53	26.23	157.33	51.99
	NA	621.90	48.34	622.98	58.35	552.23	61.38
	DOPAC	71.25	8.70	51.90	8.21	59.68	12.98
	HVA	165.30	24.24	109.93	21.03	173.08	32.25
	5HT	923.28	35.46	867.73	56.99	890.95	19.16
	5HIAA	497.30	11.64	496.30	36.33	344.53	72.45

Table 6.5. Analysis of data obtained during biogenic amines measurement in hippocampus of female and male TGT genetrapp mice. Statistical analysis of data obtained during measurement of biogenic amines in hippocampus of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.1.2 C&D).

A.		Female					
		Mean	SEM	Mean	SEM	Mean	SEM
Rest of Brain	Biogenic Amines						
	L-DOPA	85.43	10.45	77.40	9.61	81.03	17.34
	DA	628.25	39.24	557.83	65.63	497.45	15.19
	NA	467.95	62.06	422.08	20.15	319.55	67.37
	DOPAC	141.15	12.31	168.20	8.47	134.80	1.08
	HVA	395.20	44.95	457.40	55.39	321.33	24.18
	5HT	474.00	50.73	436.70	16.70	395.65	14.38
	5HIAA	296.50	45.02	287.90	27.19	215.53	14.06

B.		Male					
		Mean	SEM	Mean	SEM	Mean	SEM
Rest of Brain	Biogenic Amines						
	L-DOPA	7.80	0.47	6.35	0.68	8.43	0.56
	DA	477.23	81.84	511.15	78.15	482.23	33.00
	NA	365.60	28.17	373.53	26.80	377.60	22.50
	DOPAC	118.25	9.36	119.83	12.87	127.73	8.91
	HVA	334.80	15.34	270.68	16.75	299.38	15.22
	5HT	413.88	12.18	423.75	34.85	430.53	9.08
	5HIAA	233.40	10.23	195.25	14.20	234.45	15.12

Table 6.6. Analysis of data obtained during biogenic amines measurement in rest of brain samples of female and male TGT genetrapp mice. Statistical analysis of data obtained during measurement of biogenic amines in rest of brain samples of female and male wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.1.1 E&F).

FEMALE				MALE			
A.				B.			
5HIAA/5HT				5HIAA/5HT			
Brain Regions	+/+	+/Gt	Gt/Gt	Brain Regions	+/+	+/Gt	Gt/Gt
Cerebellum	0.79	0.79	0.59	Cerebellum	0.51	0.67	0.54
Frontal Cortex	0.40	0.40	0.37	Frontal Cortex	0.36	0.37	0.34
Hypothalamus	0.53	0.54	0.50	Hypothalamus	0.51	0.41	0.42
Striatum	0.87	0.67	0.59	Striatum	0.57	0.57	0.75
Hippocampus	0.78	0.82	0.72	Hippocampus	0.54	0.57	0.38
Rest of Brain	0.62	0.70	0.54	Rest of Brain	0.56	0.44	0.55

C.				D.			
DOPAC/DA				DOPAC/DA			
Brain Regions	+/+	+/Gt	Gt/Gt	Brain Regions	+/+	+/Gt	Gt/Gt
Cerebellum	3.07	7.12	9.01	Cerebellum	6.10	0	3.94
Frontal Cortex	0.20	0.23	0.21	Frontal Cortex	0.13	0.09	0.13
Hypothalamus	1.30	0.40	0.30	Hypothalamus	0.39	0.41	0.46
Striatum	0.08	0.09	0.9	Striatum	0.10	0.09	0.08
Hippocampus	1.54	0.81	1.084	Hippocampus	0.82	0.39	0.42
Rest of Brain	0.22	0.31	0.27	Rest of Brain	0.26	0.24	0.27

Table 6.7 Metabolite to precursor ratios for dopamine and serotonin in fine brain sections wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Tables representing 5HIAA/5HT ratio in female (A) and male (B) wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice. Table representing DOPAC/DA ratio in female (C) and male (D) wild-type, heterozygous and homozygous TGT gene trap mice.

Table 6.8 Metabolite to precursor ratios for dopamine and serotonin in fine brain sections wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Tables representing HVA/DA ratio in female (A) and male (B) wild-type (+/+), heterozygous (+/Gt) and homozygous (Gt/Gt) TGT gene trap mice. Table representing DOPAC+HVA/DA ratio in female (C) and male (D) wild-type, heterozygous and homozygous TGT gene trap mice.

A.	NA (ng/g)		DA (ng/g)		DOPAC (ng/g)		HVA (ng/g)		5HT (ng/g)		5HIAA (ng/g)	
	C	WT	C	WT	C	WT	C	WT	C	WT	C	WT
CE	750	760	200	160	250	50	300	100	400	290	370	230
FC	750	740	1200	950	800	200	750	810	820	755	600	295
HT	2600	3900	900	700	800	290	900	795	1350	2600	1000	1370
ST	550	600	17000	18000	3400	1450	2500	4400	1200	1000	610	800
HC	1050	860	450	185	300	85	500	215	1250	950	810	750

B.	NA (ng/g)		DA (ng/g)		DOPAC (ng/g)		HVA (ng/g)		5HT (ng/g)		5HIAA (ng/g)	
	C	WT	C	WT	C	WT	C	WT	C	WT	C	WT
CE	750	475	200	15	250	51	300	80	400	360	370	180
FC	750	525	1200	1180	800	150	750	700	820	650	600	220
HT	2600	2200	900	490	800	200	900	625	1350	1880	1000	990
ST	550	390	17000	11500	3400	1100	2500	2990	1200	825	610	500
HC	1050	625	450	100	300	75	500	160	1250	925	810	500

Table 6.9. Approximate biogenic amine content in different brain areas of C57Bl/6 mice reported in literature [176] and wild-type mice used in experiment. Each value represents an average mean number. *CE*, cerebellum; *FC*, frontal cortex; *HT*, hypothalamus; *ST*, striatum; *HC*, hippocampus. *NA*, noradrenaline; *DA*, dopamine; *DOPAC*, 3,4-dihydroxyphenylacetic acid; *HVA*, homovanillic acid; *5HT*, serotonin; *5HIAA*, 5-hydroxy indolacetic acid. Distributional patterns of neurotransmitters and their metabolites are compared in female (**A.**) and male (**B.**) wild-type mice used in this study with C57Bl/6 mice of unknown gender used by Van Dam *et al* [176].

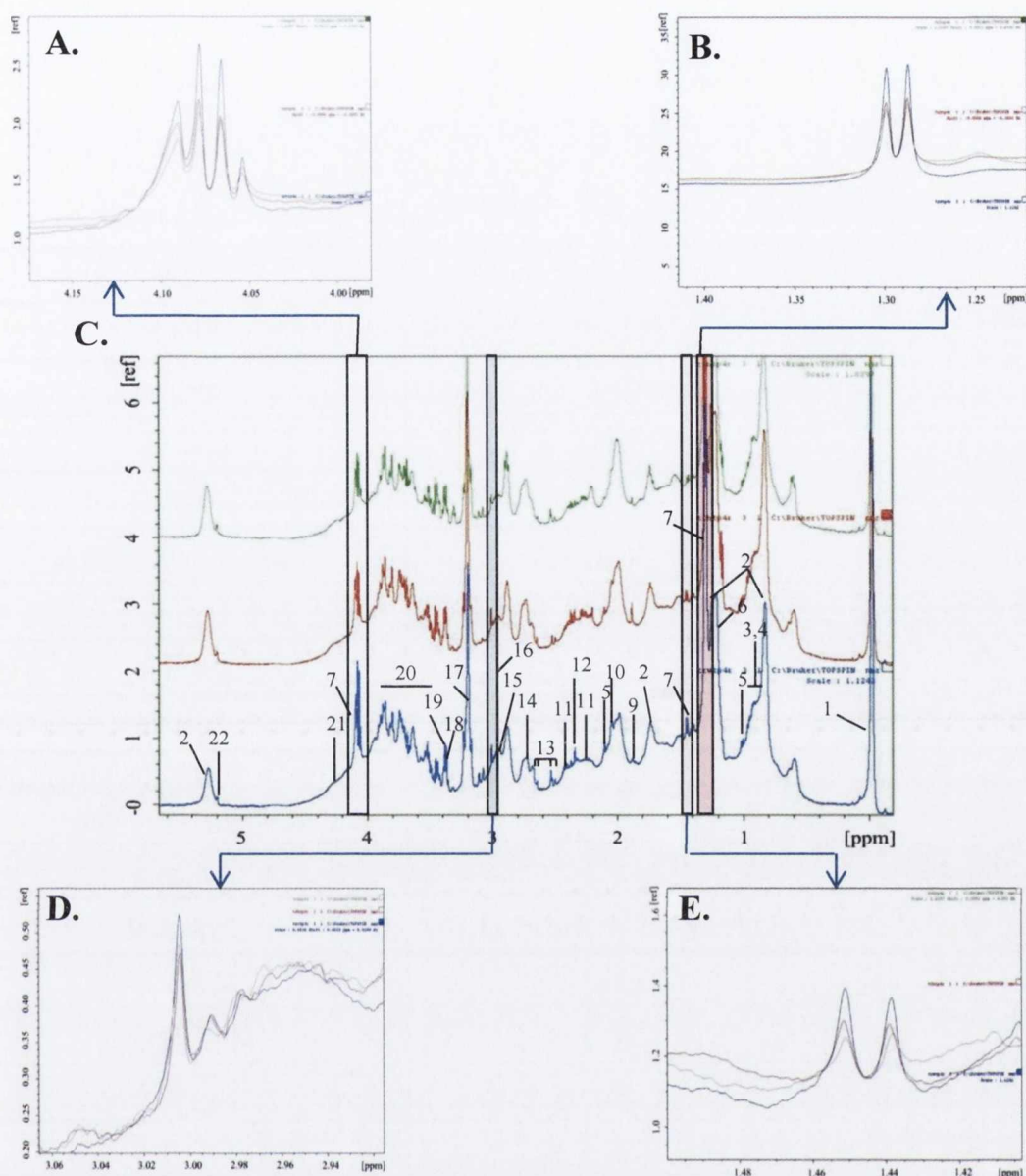


Figure 6.2 600 MHz ^1H NMR spectrum of blood plasma sample. The NMR spectra contains signals of low molecular weight metabolites as well as of larger molecules such as lipoproteins. Keys to the figure C) are given in Table 6.3. **(A, B)** ^1H NMR spectrum signals of lactate. **(D)** ^1H NMR spectrum signals of creatine. **(E)** ^1H NMR spectrum signals of alanine.

Key	Metabolites
1	TSP
2	Lipids
3	Isoleucine
4	Leucine
5	Valine
6	3-D-hydroxybutyrate
7	Lactate
8	Alanine
9	Acetate
10	NAC2+NAC1 (Acetyl signals from glycoproteins)
11	Glutamine
12	Pyruvate
13	Citrate
14	Mg-EDTA ₂
15	Lysine
16	Creatine
17	Phosphorylcholine
18	Proline
19	Scyllo-inositol
20	Glucose and amino acid resonances
21	Threonine
22	α -Glucose

Table 6.10 Assignment of the metabolites in plasma. The metabolite peaks were assigned by literature searches.

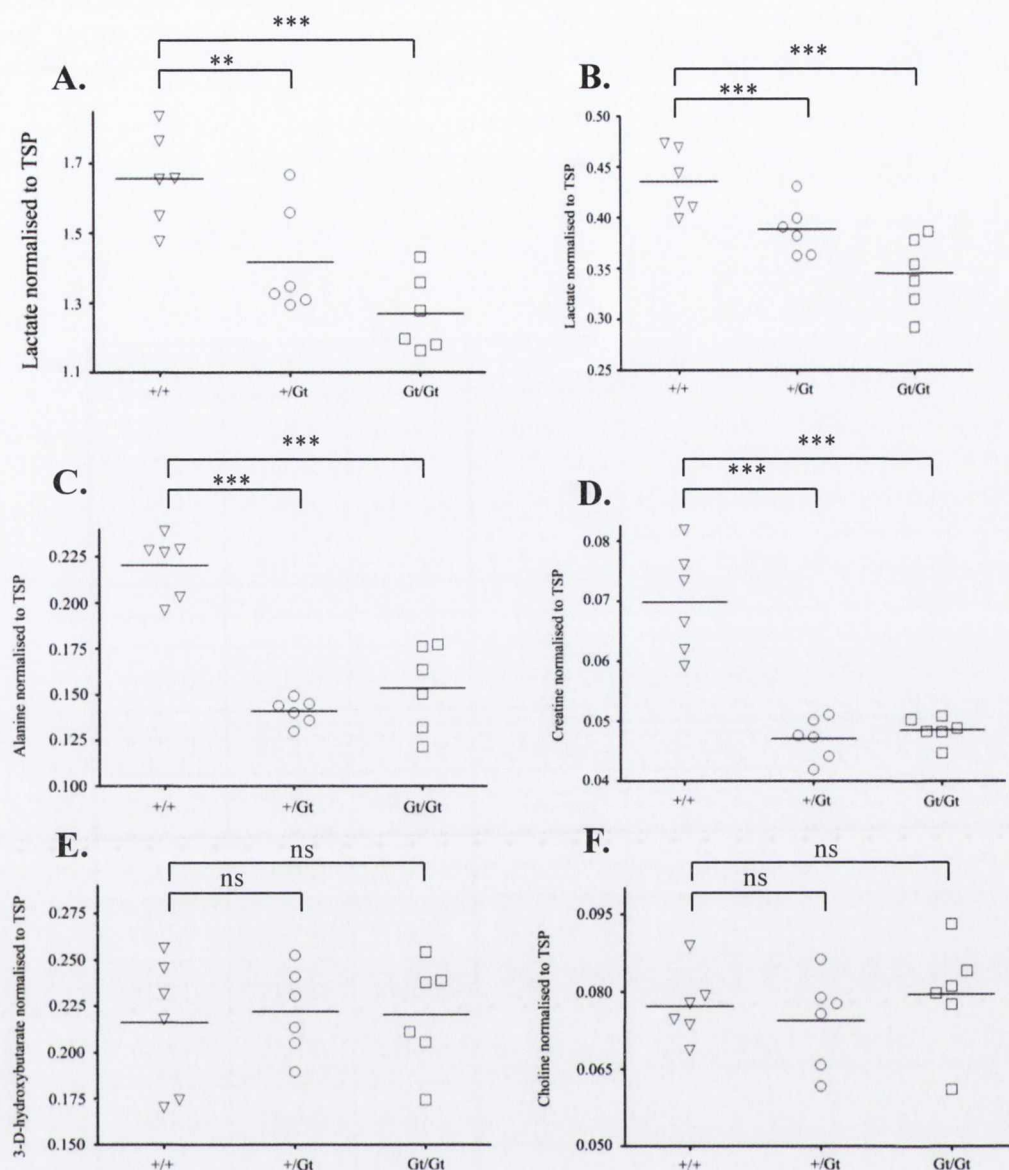


Figure 6.3 Metabolic profiling of plasma from female wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice by NMR spectroscopy. Amount of lactate present at two different regions of NMR spectra (A, B) normalised to internal TSP standard. Amount of alanine (C), creatine (D), 3-D-hydroxybutyrate (E) and choline (F) present normalised to internal TSP standard. (See Table 6.11 for Mean values, SD and SEM)

		+/+	+/Gt	Gt/Gt
Lactate A.	Mean	1.656	1.417	1.268
	SD	0.133	0.156	0.108
	SEM	0.054	0.064	0.044
Lactate B.	Mean	0.435	0.388	0.345
	SD	0.032	0.025	0.036
	SEM	0.013	0.010	0.015
Alanine	Mean	0.220	0.141	0.154
	SD	0.017	0.007	0.023
	SEM	0.007	0.003	0.009
Creatine	Mean	0.070	0.047	0.049
	SD	0.009	0.003	0.002
	SEM	0.004	0.001	0.001
3-D-Hydroxybutyrate	Mean	0.216	0.222	0.220
	SD	0.036	0.024	0.029
	SEM	0.015	0.010	0.012
Choline	Mean	0.077	0.074	0.079
	SD	0.007	0.009	0.011
	SEM	0.003	0.004	0.004

Table 6.11. Analysis of data obtained metabolic profiling of plasma of wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice. Statistical analysis of data obtained in metabolic profiling of plasma of female wild-type, *Qtrt1*^{+/Gt}, and *Qtrt1*^{Gt/Gt} mice (see Figure 6.3).

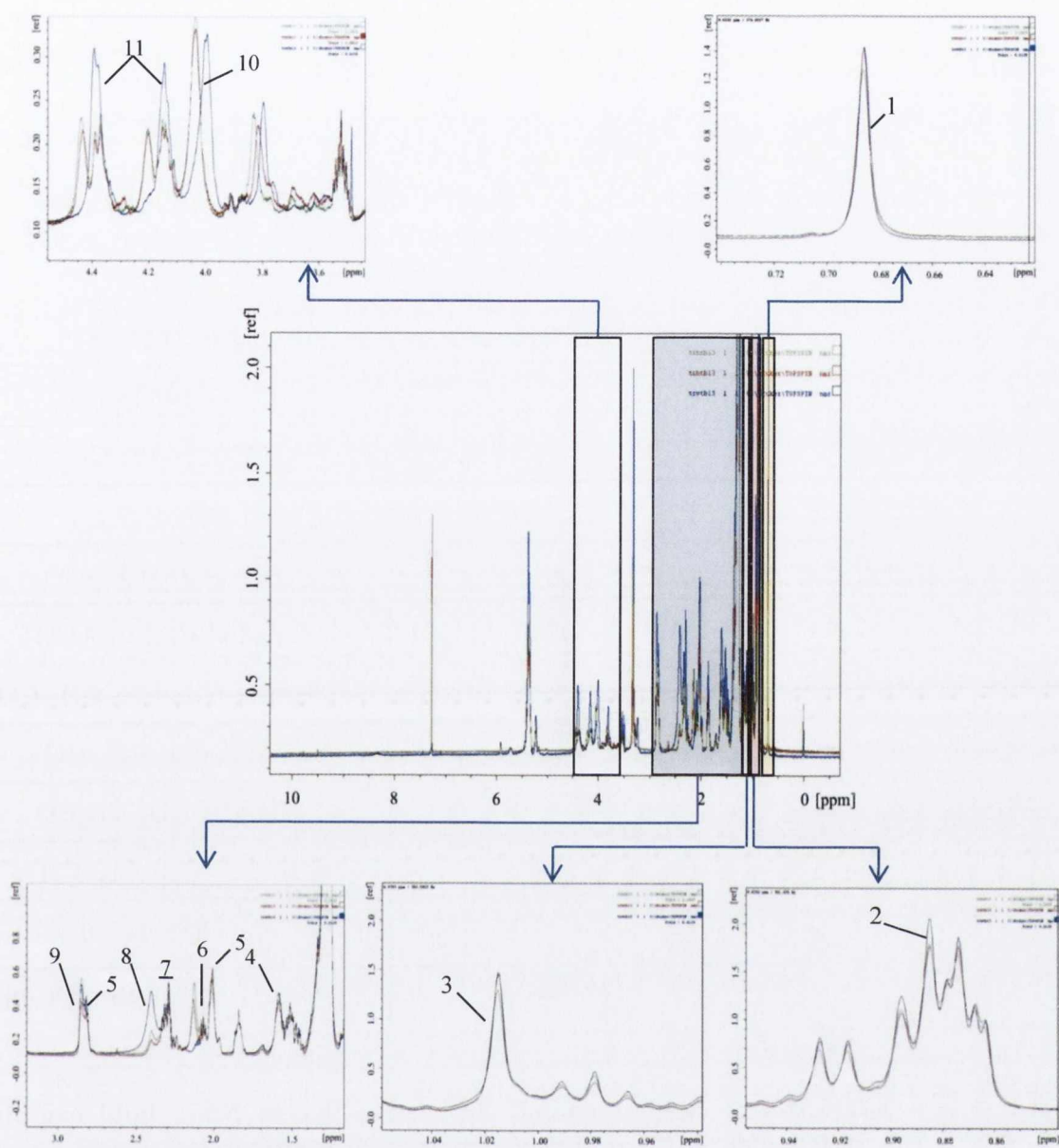


Figure 6.4 600 MHz ^1H NMR spectra of brain lipid sample of wild-type, $Qtr1^{+/Gt}$, and $Qtr1^{Gt/Gt}$ mice. The difference in the spectra are highlighted and have been magnified. Keys to the figures are given in Table 6.4

Key	Metabolites
1	Chol-18
2	$\text{CH}=\text{CH}-\text{CH}_2\text{CH}_3$
3	Chol-19
4	$\text{F}\beta$: $\text{R}-\text{CH}_2-\text{CH}_2-\text{CO}$
5	$\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}_2$
6	$\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CO}$
7	$\text{F}\alpha$: $\text{R}-\text{CH}_2-\text{CH}_2-\text{CO}$
8	$\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2\text{CO}$
9	$\text{CH}=\text{CH}-(\text{CH}_2-\text{CH}=\text{CH})_Y$
10	Gly – C3 (PC/PE)
11	Gly – C1 (DG/TG/PC/PE/PI)

Table 6.12 Assignment of the metabolites in brain lipid sample. The metabolite peaks were assigned by literature searches.

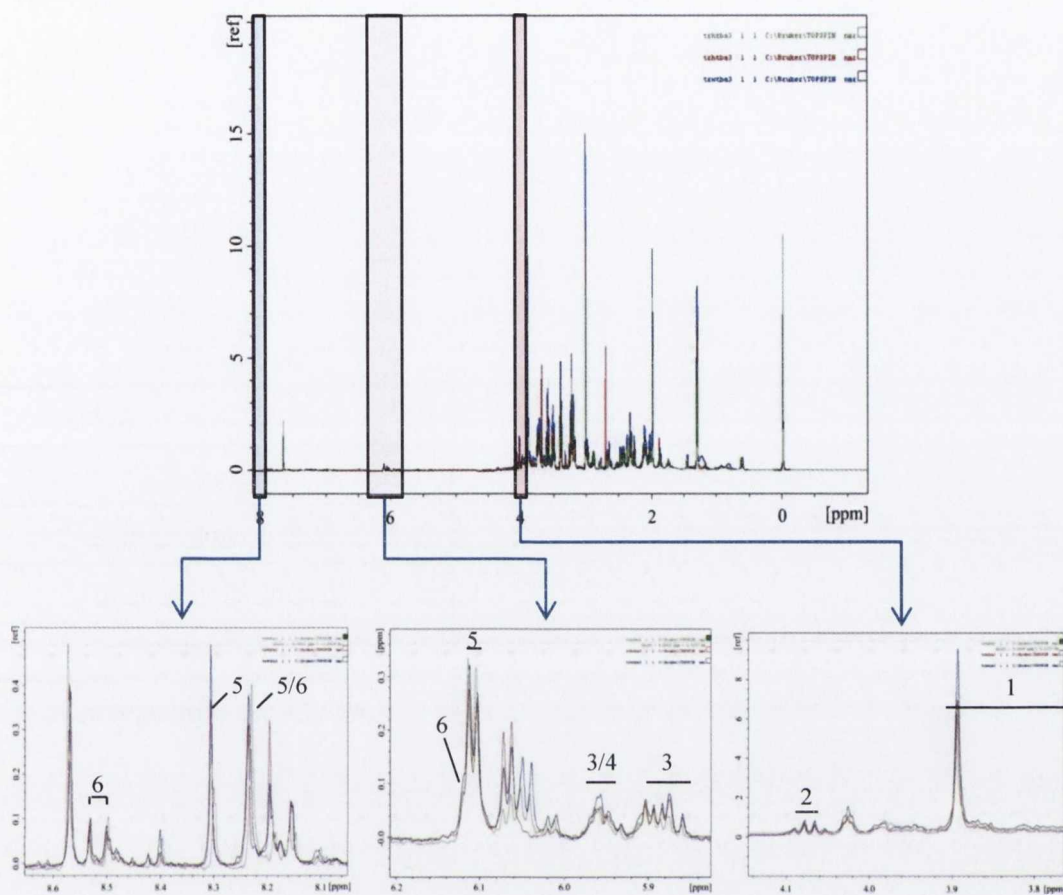


Figure 6.5 600 MHz ^1H NMR spectra of brain aqueous polar sample of wild-type, $Qtrt1^{+/Gt}$, and $Qtrt1^{Gt/Gt}$ mice. The difference in the spectra are highlighted and magnified. Keys to the figures are given in Table 6.5

Key	Metabolites
1	Sugars and Amino Acids
2	Lactate
3	Uridine triphosphate
4	Uridine diphosphate
5	Inosine
6	Adenosine-diphosphate

Table 6.13 Assignment of the metabolites in brain aqueous sample. The metabolite peaks were assigned by literature searches.

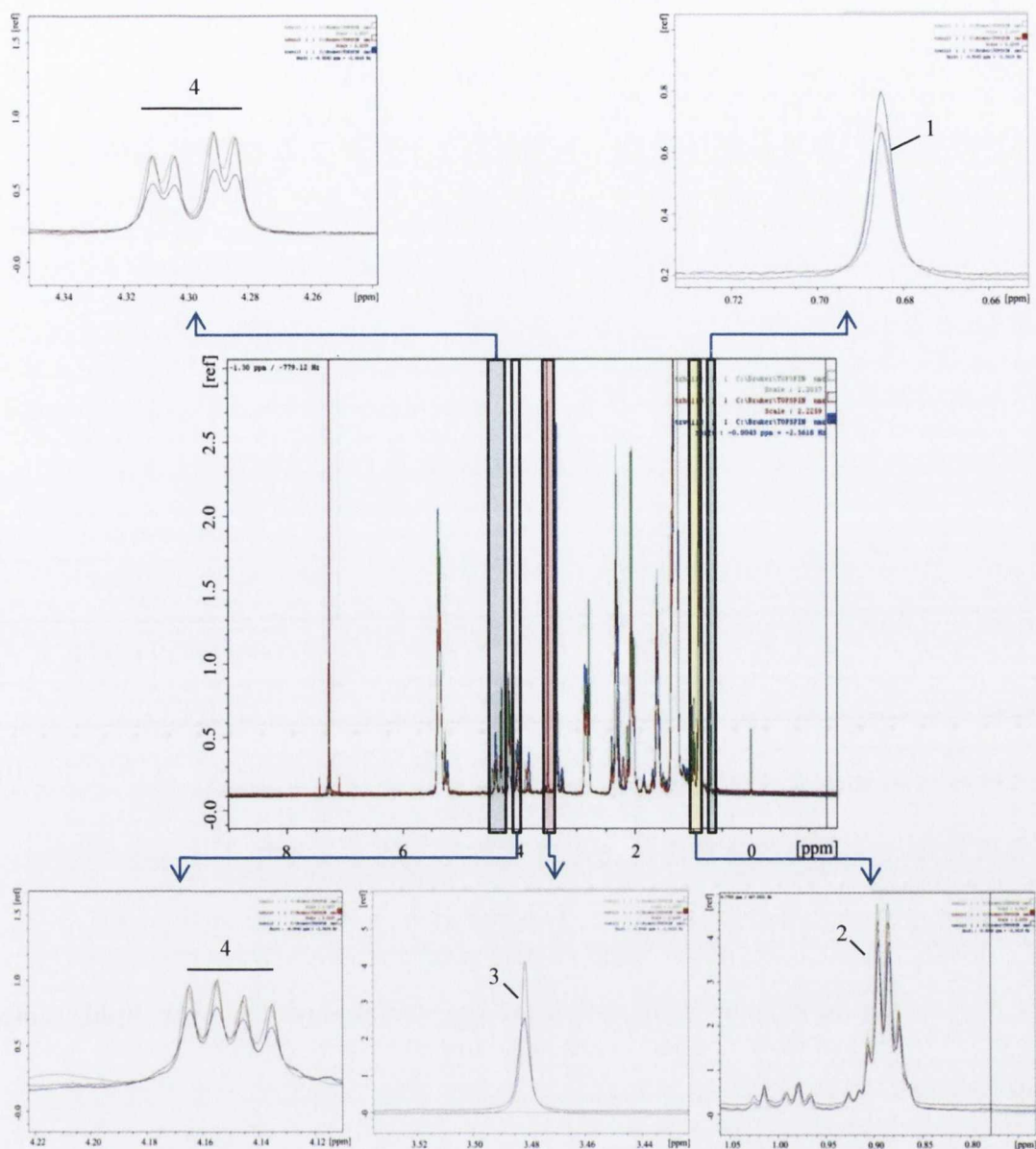


Figure 6.6 600 MHz ^1H NMR spectra of liver lipid sample of wild-type, $Qtrt1^{+/Gt}$, and $Qtrt1^{Gt/Gt}$ mice. The difference in the spectra are highlighted and magnified. Keys to the figures are given in Table 6.6

Key	Metabolites
1	Chol-18
2	CH=CH-CH ₂ CH ₃
3	CHD ₂ OD
4	Gly – C1 (DG/TG/PC/PE/PI)

Table 6.14 Assignment of the metabolites in liver lipid sample. The metabolite peaks were assigned by literature searches.

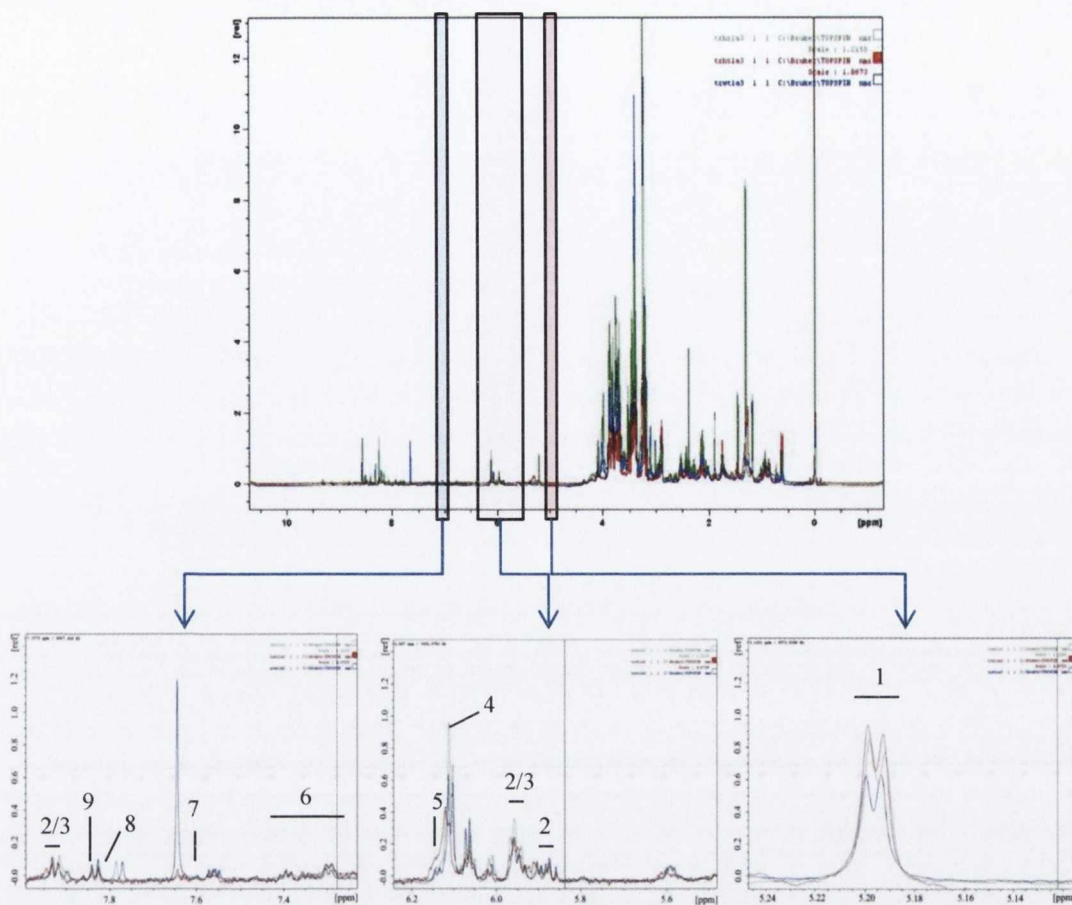


Figure 6.7 600 MHz ^1H NMR spectra of liver aqueous polar sample of wild-type, $Qtrt1^{+/Gt}$, and $Qtrt1^{Gt/Gt}$ mice. The difference in the spectra are highlighted and magnified. Keys to the figures are given in Table 6.7

Key	Metabolites
1	α -Glucose
2	Uridine triphosphate
3	Uridine diphosphate
4	Inosine
5	Adenosine-diphosphate
6	Phenylalanine
7	Nicotinurate
8	Histidine
9	Uridine

Table 6.15 Assignment of metabolites in liver aqueous sample. The metabolite peaks were assigned by literature searches.

6.3 Discussion

For the purposes of this study it was decided to look more closely at the effect of queuine in the brain. *Qtrt1^{Gt/Gt}* mice have no ability to synthesize queuosine tRNA (work done by Coilin Boland) and it was shown that TGT genetrap mice have impaired tyrosine production, an accumulation of BH₂ and altered behavioural traits. Since the amino acid tyrosine and BH₄ are essential components in catecholamine and indoleamine neurotransmitter biosynthesis, which may account for behavioural changes [195, 246], it was decided to compare the levels of biogenic amines in six different sections of the female and male brain, cerebellum (CE), frontal cortex (FC), hypothalamus (HT), striatum (ST), hippocampus (HC) and the rest of brain (RB), which was left over from sectioning. This comparison showed that the level of biogenic amines vary between female and male mice of the three genotypes.

Dopamine and serotonin are rapidly metabolised to form HVA and 5HIAA, and the measurement of these metabolites may be used to estimate the overall turnover of neurotransmitters within the brain. In female and male *Qtrt1^{Gt/Gt}* mice the formation of serotonin and its metabolite 5HIAA seem to be compromised when compared to wild-type mice but not to the same extent. In female mice an overall decrease in the ratio of serotonin turnover was observed which might mean that the release of this neurotransmitter is not as efficient in *Qtrt1^{Gt/Gt}* female mice when compared to wild-type mice or that there is an overall decreased activity of the whole serotonergic system. However, in male mice the levels of serotonin and its metabolite vary from region to region

Variability in the levels of the rest of biogenic amines produced from tyrosine metabolism was found to be very high in female and male wild-type and TGT genetrap mice and further detailed studies are required to generate a clear understanding of queuine's role in the brain. The differences in the levels of biogenic amines observed between three genotypes may account for behavioural differences in *Qtrt1^{Gt/Gt}* female mice, however abnormalities in biogenic amine production in male mice does not relate to the lack of any behavioural change.

Abnormalities in the levels of dopamine and serotonin metabolites can be detected when their biosynthesis is disturbed as observed in a number of human diseases. This

usually arises from genetic deficiencies in TH, aromatic-L-amino acid decarboxylase and enzymes involved in the synthesis and recycling of BH₄ GTPCH I, PTPS, SR and DHPR. Diagnosis of these disorders usually rely on the measurements of pterins, 5HT, L-DOPA, 5HIAA, HVA and the ratio of HVA to HIAA. The HVA is a stable end product of dopamine and reflects dopamine turnover, while 5HIAA reflects serotonin turnover.

Although the changes in the level of biogenic amines in TGT genetrap mice may cause the behavioural changes observed. The effect may be localised to one region or neuronal path in the brain. Alternatively, the changes might be too small to be accurately determined by HPLC. This behavioural differences might results from the combination of all of this changes in the level of biogenic amine together with the change in the level of metabolites in the brain that were observed by NMR analysis.

The decreases in dopamine and serotonin metabolites can be seen in several metabolic diseases such as PKU. The accumulation of phenylalanine results in inhibition of tyrosine and tryptophan hydroxylases as well as the transport of precursors across the blood brain barrier. The symptoms of genetic disorders of dopamine and serotonin metabolism usually include hypotonia in babies, seizures, dystonia, instability in temperature, tremor, psychomotor retardation if untreated and many more [247]. A number of neurological diseases are also characterised by an abnormality in biogenic amines such as narcoleptic epilepsy [246], which is a chronic and debilitating sleep disorder and is caused by accumulation of serotonin.

A decrease in the level of biogenic amines is generally due to higher activity of the MAO enzyme responsible for destruction of biogenic amines so in future it would be good idea to examine its activity in all three genotypes.

To make sure that the levels of biogenic amines obtained for our wild-type control mice are of the right levels it was decided to compare them with those found in the literature. A paper by Van Dam *et al.* [242] provided useful information in that respect. It was noticed that the levels of some biogenic amines were lower in female mice when compared to the values published by Van Dam and variability also existed in the levels of biogenic amines in male wild-type mice. An explanation for this might be the diet that their and our mice were kept on. For the purposes of this study mice were placed on chemically defined diet, while mice in Van Dam experiment received normal diet. So differences might arise from this.

It was decided to look more closely at biochemical composition of plasma, liver and brain with the aid of ^1H NMR spectroscopy. In plasma, lower levels of lactate, alanine and creatine were observed in TGT genetrap to wild-type mice. In liver and brain lipid fractions differences were noticed between genotypes in the levels of cholesterol, and unsaturated fatty acyl moieties. Liver and brain polar aqueous fractions also displayed variability in the levels of metabolites such as sugars, amino acids, lactate, inosine, adenosine-diphosphate and uridine triphosphate.

Of particular interest, TGT genetrap mice showed a notable decrease in metabolites that act as indicators of mitochondrial function, lactate, alanine and creatine when compared to wild-type. Lactate and alanine are formed from pyruvate a molecule that supplies energy to living cells through the citric acid cycle. Pyruvate can be converted to carbohydrate via gluconeogenesis, and to fatty acids through acetyl-CoA. Creatine is converted to phosphocreatine in a reaction catalyzed by creatine kinase with subsequent conversion of ATP to ADP [248]. The levels of ADP were also found to be different in polar molecule fractions of liver and brain. These results, especially the decrease in lactate, could signify that *Qtrt1*^{Gt/Gt} mice are diverting energy production towards oxidative phosphorylation by the mitochondria and away from glycolysis.

The other metabolites found to be compromised in liver and lipid fractions were unsaturated fatty acyl moieties and cholesterol. In a study carried out by Igarashi *et al.* [249] unsaturated fatty acyl group were shown to have a stimulatory role on rat adrenal mitochondrial steroidogenesis. Cholesterol on the other hand has a role in building and maintenance of cell membranes, it reduces the permeability of membrane to protons and sodium ions. However, within the cell membrane, cholesterol has other functions such as intracellular transport, cell signalling and nerve conduction [250].

In conclusion this analysis of biological fluids from wild-type and TGT genetrap mice provides us with a grander picture of changes in total biochemical composition that the absence of one gene provides. In future, it would be interesting to identify exact metabolites that differ between genotypes, to gain further knowledge and understanding of the mechanism behind these changes.

Chapter 7

Final Discussion and future work

7.1 Discussion

Queuosine has been implicated in many important physiological functions such as differentiation, development, hypoxic stress management, and most importantly to this study, tyrosine biosynthesis.

The results obtained by Marks and Farkas [64] showed an absolute requirement for queuine in the absence of tyrosine in germ-free mice. In this study it was decided to investigate if these results can be reproduced in a human cell line. Hepatoma HepG2 cells were chosen for this purpose as they were shown to contain the enzymes required for BH₄ biosynthesis and recycling pathway in addition to an active PAH enzyme [112-120].

Before the start of the investigation into the effect of queuine on tyrosine production could take place it was necessary to establish queuine deficient and sufficient growth conditions. For this purpose a new simple and straight forward method was devised to confirm the presence and absence of queuine in HepG2 cells. This method was devised on the basis of earlier methods described by Nishimura and his group [49-50, 151]. It was based on the ability of *E.coli* TGT enzyme to insert radiolabelled guanine into tRNA that is not modified by queuosine. The results obtained by this method were further confirmed by analysis of the queuine levels in HepG2 cells by LCMS in collaboration with Vimbai Chikwana (Portland State University). The present work clearly indicates that our newly devised method is working as well as LCMS method but is simpler and does not require expensive equipment and specialist expertise.

In this work it was clearly shown that tyrosine production is compromised after 30 minutes in HepG2 cells grown in queuine deficient conditions, while phenylalanine uptake was found to be linear to the amount of phenylalanine. The result correlate with those obtained by Marks and Farkas [64] in germ-free mice. The conclusion from this experiment can be made that in human HepG2 cells queuine is essential for the proper production of tyrosine and by extension that humans require queuine for tyrosine biosynthesis.

It was also noted that queuine is somewhat structurally related to BH₄ as they are both synthesized from GTP. However, queuine or queuosine modified tRNA does not bind to or affect the activity of the PAH enzyme, as demonstrated by our kinetic analysis using recombinant PAH. By carrying out *in vitro* assays to measure tyrosine production in HepG2 cells it was also shown that queuine does not affect PAH protein post-transcriptionally or as a result of a build-up of a toxic metabolite.

In the study carried out by Reyniers *et al.* [39] it was shown that germ-free mice appear to be completely normal in the absence of queuosine-tRNA, so in order to understand the specific role that queuine is playing, TGT genetrap mice were created. The initial characterisation of TGT genetrap mice showed an increase in body weight when compared to wild-type. Interestingly, further measurements of organ weight displayed a decrease in heart and spleen weight in *Qtrt1^{Gt/Gt}* mice when compared to wild-type. Previously in the study carried out by Coovadia *et al.* [251] it was shown that placing mice on amino acid restricted diets results in a decrease of some organ weights including spleen. The possibility exists that an alteration in the levels of amino acids exists, especially tyrosine, occurs in these TGT genetrap mice and this may result in the body and organ weight changes. It should be noted that this study did not observe significant changes in the basal level of tyrosine in serum but the differences may be too subtle to detect using HPLC or perhaps individual cell types could be experiencing decreased tyrosine, for example immune cells of the spleen.

The generation of TGT genetrap mice allowed us to examine the effect of queuine on tyrosine biosynthesis in living organisms. Tyrosine biosynthesis was shown to be deficient in TGT genetrap mice over time after administration of exogenous phenylalanine when compared to wild-type. This agreed with the results obtained in HepG2 cells. Interestingly, in mice tyrosine biosynthesis was found to be compromised over time in both heterozygotes and homozygotes. This could be explained by results from our laboratory showing that 6-8 week heterozygote mice are deficient in queuosine-tRNA. It is suspected that the TGT-genetrap allele, which makes an inactive fusion protein, is acting in a dominant-negative fashion to sequester the TGT partner protein Qv1 away from the normal wild-type protein resulting in greatly reduced TGT activity in heterozygotes.

Again this difference in tyrosine biosynthesis over time was not due to a difference in endogenous PAH protein specific activity or expression, nor was it due to the difference in the expression level of TH protein responsible for tyrosine metabolism. However, the possibility still existed that a metabolite of unknown identity was accumulating in animals deficient in queuosine modified tRNA. It was possible to rule out this possibility by performing an *in vitro* PAH assay in liver cell extracts where no difference was observed between wild-type and genetrapp animals. As reduced BH₄ was being added in excess to these *in vitro* PAH assays the possibility that the loss of queuosine modified tRNA could be affecting BH₄ levels in liver could not be addressed.

It was decided to look at the levels of BH₄ and its metabolites directly. Deficiencies in BH₄ pathways can be found in number of diseases including PKU, diabetes, coronary heart disease, as well as Parkinson's disease, depression, autism and Alzheimer's disease and can lead to neurological disorders such as Dopa-responsive dystonia [91, 101]. This study demonstrated a striking increase in BH₂ levels in urine, liver and plasma of TGT genetrapp mice when compared to wild-type. It was also found that the level of total biopterin was higher in urine and the level of BH₄ was lower in plasma. Increased BH₂ levels can be found in patients with onset of diabetes and other chronic vasoinflammatory conditions [121]. Proportionally reduced ratio of BH₄/BH₂ can be found in patients with chronic renal failure with increase in severity of renal failure [122] and reduced BH₄/BH₂ ratio can be observed in patients with insulin resistance [123]. Another disease that is characterised by increased levels of biopterin and BH₂ in CSF is sepiapterin reductase deficiency [85].

The measurement of the specific activity of DHFR enzyme, responsible for regeneration of BH₄ from BH₂, showed no difference between genotypes so this difference in the levels of pterins does not arise from inactivity of this protein. Another possibility is that the expression or activity of the DHPR enzyme may be affected as BH₂ can arise from mutation of this protein, which is essential in the recycling of BH₄. A further possibility is that BH₄ is being oxidised by reactive oxygen species or peroxynitrite to BH₂ [161]. Recent studies have demonstrated that low BH₄ levels in addition to high BH₂ levels can uncouple eNOS in the endothelium, promoting the generation of reactive oxygen species and accelerating the production of BH₂, which only exacerbates the uncoupling [119].

Another interesting study showed that various pteridine analogues can inhibit formation of queuosine-tRNA in mammalian tissue culture system but this is not due to pterin being incorporated into tRNA [160]. The accumulation of BH₂ observed in this study could in theory have an inhibitory effect on the TGT enzyme resulting in the delayed queuosine-tRNA formation in older mice (12-18 weeks) and the absence of queuosine modification in younger mice (6-8 weeks), which would explain why tyrosine biosynthesis over time after administration of phenylalanine was compromised in both heterozygous and homozygous TGT genetrapped mice.

The deficiencies in regeneration of recycling of BH₄ pathways are likely to lead to neurological and behavioural disorders and indeed the investigation into the impact of queuosine modification in tRNA on mouse behaviour and metabolism yielded a number of interesting results. Behavioural studies carried out in home cage and open field environments showed that only female TGT genetrapped mice displayed differences in behaviour and had lower levels of anxiety together with higher level of exploration. In studies carried out previously it was shown that mice that are deficient in dopamine are severely hypoactive, while disruption in dopamine transporter activity results in hyperactivity [177]. In a paper by Andersen *et al.* [188] behavioural changes were noticed in rats with experimental PKU, rats with PKU were found to be more active in the open field experiments when compared to the controls. This coincided with clinical characteristics of hyperactivity in PKU patients. So TGT genetrapped mice seem to display behavioural differences that are characteristic of PKU patients and might have disruption in dopamine transporter activity.

The analysis of the biogenic amines displayed a number of differences in the amounts present in different genotypes. One major difference observed was in female TGT genetrapped mice the whole serotonergic system seemed to be affected. Though no other drastic changes in biogenic amines were observed the small differences might be sufficient to cause the behavioural differences, which may occur due to effect on one brain region or neuronal path or combination of both. Further more detailed studies need to be carried in order to clarify the results obtained.

An investigation into the biochemical composition of plasma, liver and brain with the aid of ¹H NMR spectroscopy displayed differences in the levels of metabolites present. The majority of metabolites affected seem to be involved in energy metabolism within

the cell. The levels of glucose, lactate and alanine, three important molecules in energy production, were found to be significantly decreased. Creatine, a molecule that helps supply energy to muscle cell, was also found to be lower in TGT genetrapped mice when compared to wild-type. During the conversion of creatine to phosphocreatine, ATP is converted to ADP, another molecule that was found to be affected. Differences were also observed in the levels of cholesterol, a molecule used in building and maintaining of cell walls as well as having functions in intracellular transport, cell signalling and nerve conduction, and unsaturated fatty acyl moieties. These differences in metabolites show the importance of queuine in maintaining proper bioenergetics within the cell. Interestingly, the decreases in the metabolites required for energy production does not appear to have any overt adverse effects on TGT genetrapped mice, including their growth or reproduction.

In conclusion this study shows that the bacterial derived queuine molecule impacts on multiple physiological functions in eukaryotes; the production of tyrosine over time from phenylalanine, the maintenance of reduced BH_4 , the normal behaviour of female animals and energy substrate metabolism.

7.2 Future work

This work produced many unexpected results and a more in depth study into the effect of queuosine-tRNA modification is warranted. Although queuine deficiency leads to defects in tyrosine production and accumulation of oxidized BH₄ the underlying cause of these changes is unclear. It may be informative to examine the enzymes of the BH₄ recycling pathway or determine if queuine deficient cells experience elevated levels of oxidative stress. It would be of interest to analyse the plasma of animal that have been co-administered BH₄ and phenylalanine to see if the effect of tyrosine impairment can be rescued or whether additional BH₄ metabolites accumulate. In future work, it would also be beneficial to use a tracer phenylalanine in order to make sure that all of the tyrosine produced actually comes from phenylalanine that was administered to the mice.

One of the most important and critical studies carried out in this field was done by Marks and Farkas [64]. The experiment where tyrosine withdrawal from the diet of queuine deficient germ-free mice results in their death is one of the most significant in this field. However this experiment has never been repeated under the same conditions and in future it would be useful to see if these results can be obtained again.

A more in detail study needs to be carried out into the effect of queuine on neurotransmitter production and behaviour. Perhaps, the most appropriate starting point would be a more detailed behavioural analysis as it is non-invasive and can provide clues to the underlying defects. Simple tests such as hearing or sight tests can be carried out. Examining the animal reaction to a loud noise such as clap can be sufficient. A simple test for vision would involve determining whether an animal stops at the edge of the table. Visual acuity can also be tested by a platform test in the Morris water maze. Coordination can be measured by carrying out rotorod test, which is a test that is usually carried out in mutant mice with impaired cerebral function [252]. Learning and memory tests can also be carried out. Tests to measure attention and working memory can be carried out by radial arm maze test [253]. The Morris water maze can be used to measure spatial learning of these mice which is critically dependent on hippocampal function [254]. So tests can be devised to measure the function of different regions in the brain to get a better understanding of which part of the brain is affected and the likely neurotransmitters involved.

The effect of queuosine-tRNA deficiency on metabolism can also be studied in more detail. By looking at the results already obtained it is obvious that the major changes occurred in metabolites involved in the bioenergetic status of the cell. A more in depth analysis of these metabolites needs to be carried out. It may be possible to identify the metabolites that were changed in lipid and polar fractions of brain and liver organs of TGT genetrap mice when compared to wild-type.

Chapter 8

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