Gene Transfer as a Potential Treatment for Tetrahydrobiopterin Deficient States

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ABSTRACT

Tetrahydrobiopterin (BH_4) is an essential cofactor for dopamine (DA), noradrenaline (NA), serotonin and nitric oxide (NO) synthesis in the brain. Inborn errors of BH_4 metabolism including GTP cyclohydrolase 1 (GTP-CH) deficiency are debilitating diseases in which BH_4, DA, 5-HT and NO metabolism are impaired. Current treatment for these disorders is typically monoamine replacement +/- BH_4. Whilst correction of the primary defect is the ideal, BH_4 treatment is problematic as it is expensive and inefficacious. One approach to treat BH_4 disorders is to use gene therapy as a more permanent, effective alternative. In this thesis the potential of gene therapy in an animal model of partial BH_4 deficiency, the *hph-1* mouse, was examined. These mice show many neurochemical similarities associated with BH_4 deficient states, including impaired BH_4 (-69%), DA (-14%), NA (-23%), serotonin turnover (-55%) and NO metabolites in the brain. In cultured astrocytes from *hph-1* mice BH_4 was significantly lower than wild type (-53%), and produced less BH_4 (-89%) and NO metabolites (-64%) when stimulated with lipopolysaccharide (LPS) plus interferon-γ (IFN-γ), stimuli that increase GTP-CH and iNOS expression. When *hph-1* astrocytes were infected with a recombinant adenovirus encoding human GTP cyclohydrolase (AdGCH), concentration-dependent increases in BH_4 levels were observed, with just 1 virus particle per 10 cells resulting in 50-fold increases in BH_4. AdGCH can upregulate the impaired NO production observed in *hph-1* astrocytes following stimulation with LPS + IFN-γ, although only if BH_4 was increased prior to stimulation. Examination of the molecular mechanisms behind the impaired NO production in LPS + IFN-γ stimulated cells revealed that iNOS dimerisation is attenuated in *hph-1* astrocytes when compared wild type (-84%), and could be increased to wild type levels when cells were pre-treated with AdGCH. Analysis of total iNOS protein expression revealed no difference between wild type and *hph-1*. These results raise the possibility that gene therapy could be used as a corrective solution for tetrahydrobiopterin deficient states.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
6PTP	6-pyruvoyl-tetrahydropterin
AADC	Aromatic L-amino acid decarboxylase
AAV	Adeno-associated viruses
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADA	Adenosine deaminase
AdGCH	Adenovirus containing recombinant human GTP-cyclohydrolase
AdeGFP	Adenovirus containing recombinant enhanced green fluorescent protein
AIDS	Acquired Immune Deficiency Syndrome
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APS	Ammonium persulfate solution
AR	Aldose reductase
Arg	Arginine
BH_2	7,8-dihydrobiopterin
BH_4	5,6,7,8-Tetrahydrobiopterin
BIODEF	International Database of Tetrahydrobiopterin Deficiencies
BIOMDB	International Database of Mutations Causing Tetrahydrobiopterin Deficiencies
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAR	Coxsackievirus and adenovirus receptor
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine 3',5'-monophosphate
Cit	Citrulline
CR	Carbonyl reductase
CSF	Cerebrospinal fluid
DA	Dopamine
DAHP	2,4-diaminohydroxypyrimidine
DAT	Dopamine transporter
DETAPAC	Diethylenetriaminepentaacetic acid
DHFR	Dihydrofolate reductase
DHNTP	7,8-dihydroneopterin triphosphate
DHPR	Dihydropteridine reductase
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle's Medium
$DMPH_4$	Dimethyltetrahydropterin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease 1
Dopac	3,4-dihydroxy-phenylacetic acid
DRD	Dopa responsive dystonia
DTE	Dithioerythritol
DTT	Dithiothreitol
EBSS	Earles' Balanced Salt Solution

ECD	Coulometric electrochemical detection
EDRF	Endothelial derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EIA	Competitive enzyme immunoassay
ELISA	Enzyme linked immunosorbant assay
eNOS	Endothelial NOS
ENU	N-ethylnitrosourea
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FMN	Flavin mononuleotide
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCH	Gene for GTP-CH
GCH-Tg	GTP-CH transgenic mice
GFRP	GTP cyclohydrolase feedback regulatory protein
GSH	Glutathione
GTP	Guanosine 5'-triphosphate
GTP-CH	GTP cyclohydrolase 1
HA	Haemagglutinin
HBSS	Hanks' Balanced Salt Solution
HD-Ads	Helper-dependent adenoviruses
HEC's	Human endothelial cells
HIV	Human immunodeficiency virus
HPA	Hyperphenylalaninaemia
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HVA	Homovanillic acid
ICV	Intracerebroventricular
IFN-γ	Interferon γ
IL-β	Interleukin-β
iNOS	Inducible NOS
L-dopa	L-3,4-dihydroxyphenylalanine
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NIL	N6-iminoethyl-L-Lysine
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MEM	Minimum essential medium
MHPG	3-methoxy-4-hydroxyphenylglycol
MOI	Multiplicity of infection
MPP^{+}	1-methyl-4-phenylpyridinium
MRP1	Multi-drug resistance protein type 1
MTHFR	Methylenetetrahydrofolate reductase
mtNOS	Mitochondrial NOS
NA	Noradrenaline
NADH	β-nicotinamide adenine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide phosphate
NHA	N-^{G} Hydroxy-L-arginine
nNOS	Neuronal NOS
NO	Nitric oxide

NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NOx	Nitrite and nitrate
NSB	Non-specific binding
$ONOO^-$	Peroxynitrite
OTC	Ornithine transcarbamylase
PAGE	Polyacrylamide gel electrophoresis
PAH	Phenylalanine hydroxylase
PBS-T	10% phosphate-buffered saline with 0.1% Tween
PC12	Pheochromocytoma cells
PCA	Perchloric acid
PCD	Pterin 4a-carbinolamine dehydratase
PCR	Polymerase chain reaction
PD	Parkinson's disease
pfu	Plaque forming units
PI3K	Phosphoinositide 3-kinase
PKU	Phenylketonuria
PMSF	Phenylmethylsulforyl fluoride
PTPS	6-pyruvoyl-tetrahydropterin synthase
PTS	Gene for PTPS
Pts(-/-)	6-pyruvoyltetrahydropterin deficient mice
q-BH_2	Quinonoid-dihydrobiopterin
q-$DMPH_2$	Quinonoid-dimethyldihydropterin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sep	L-sepiapterin
SNAP	S-Nitroso-N-acetyl-*dl*-penicillamine
SNc	Substantia nigra pars compacta
SPR	Gene for SR
SR	Sepiapterin reductase
SR(-/-)	Sepiapterin reductase deficient mice
TA	Total Activity
TEMED	N,N,N',N'-Tetramethylethylenediamine
TH	Tyrosine hydroxylase
TRH	Tryptophan hydroxylase
VMAT2	Vesicular monoamine transporter type 2
WT	Wild type
µA	µamps

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Chapter 1

Introduction

1. INTRODUCTION

1.1 Pterins and pteridines

Tetrahydrobiopterin (BH_4) belongs to a large class of compounds known as pteridines, and within a subdivision of this group named pterins. Pteridines are natural structurally diverse compounds that are involved in the biosynthesis of vitamins and cofactors. The structures of pteridines are based around a bicyclic nitrogen ring system, of which pterins are 2-amino-4-oxo derivatives (figure 1.1). Pterins can be further divided into two groups. Conjugated pterins, also known as folates, and unconjugated pterins that have a substitution that occurs mainly at the 6 position of the ring nucleus, which includes BH_4 (Pfleiderer, 1984). The name pterin was first applied by F. Gowland Hopkins in 1889 (Hopkins, 1889), when isolating a yellow pigment from the wing of the common English brimstone butterfly. The name pterin arises from the Greek word *pteron*, meaning wing.

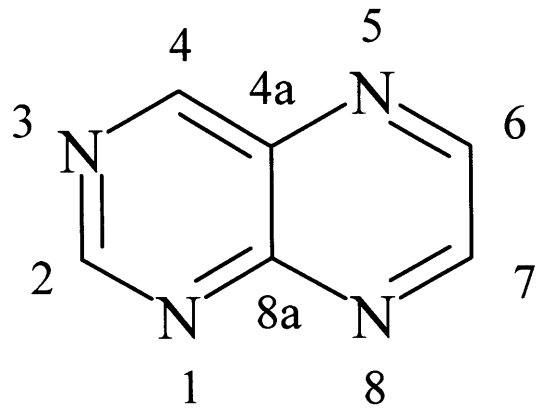
1.2 Tetrahydrobiopterin

Biopterin, the fully oxidised form of BH_4, was discovered independently and almost simultaneously by two groups of investigators. It was identified both as a growth factor for the protozoon *Crithidia fasciculata* (Patterson *et al*, 1956) and as an eye pigment in the mutant phenotype of *Drosophila melanogaster* (Forrest and Mitchell, 1954). The structure of biopterin is a dihydroxypropyl side chain attached to the pterin ring at position 6, and is fully oxidised in this form. Biopterin is partially reduced at positions 7 and 8 in the dihydrobiopterin (BH_2) form, and fully reduced as BH_4 (figure 1.2). However, only the 5,6,7,8-tetrahydro form is biologically active (Blau *et al*, 2001).

1.3 *De novo* biosynthesis of tetrahydrobiopterin

BH_4 is synthesized from the purine guanosine 5'-triphosphate (GTP)(figure 1.3). The first step in the pathway involves the opening of the ribose ring, and then purine moieties of GTP, followed by the incorporation of a carbon from the ribose into the purine ring (Fukushima and Shiota, 1974, Bracher *et al*, 1998), which results in formation of the pterin ring structure. This reaction, known as an Amadori rearrangement, is the initial rate-limiting step in the pathway, which is catalysed by GTP cyclohydrolase 1 (GTP-CH)(EC 3.5.4.16) and produces 7,8-dihydroneopterin

Pteridine



Pterin

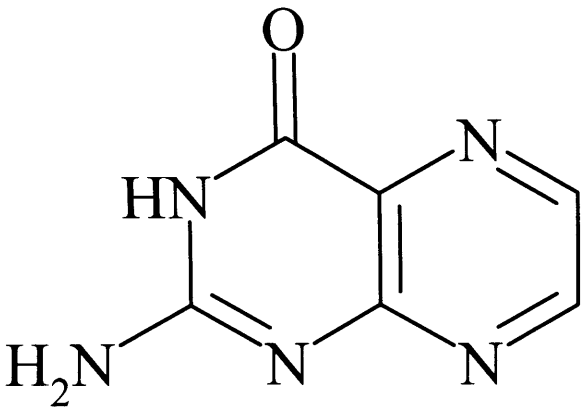
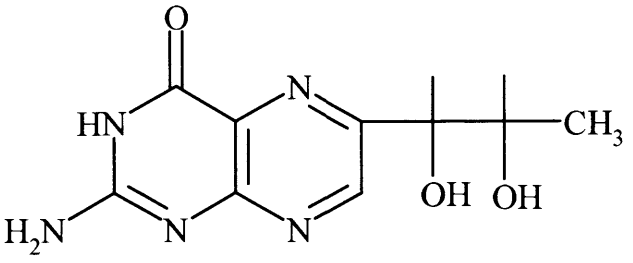
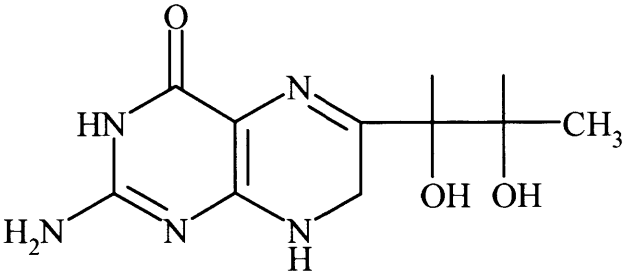


Figure 1.1. The structures of pteridine and pterin.

Biopterin



7,8-dihydrobiopterin



Tetrahydrobiopterin

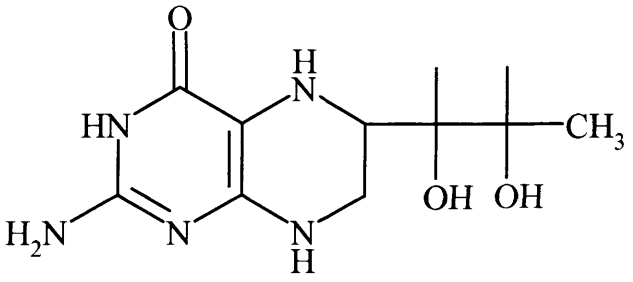


Figure 1.2. Structures of the oxidation states of tetrahydrobiopterin.

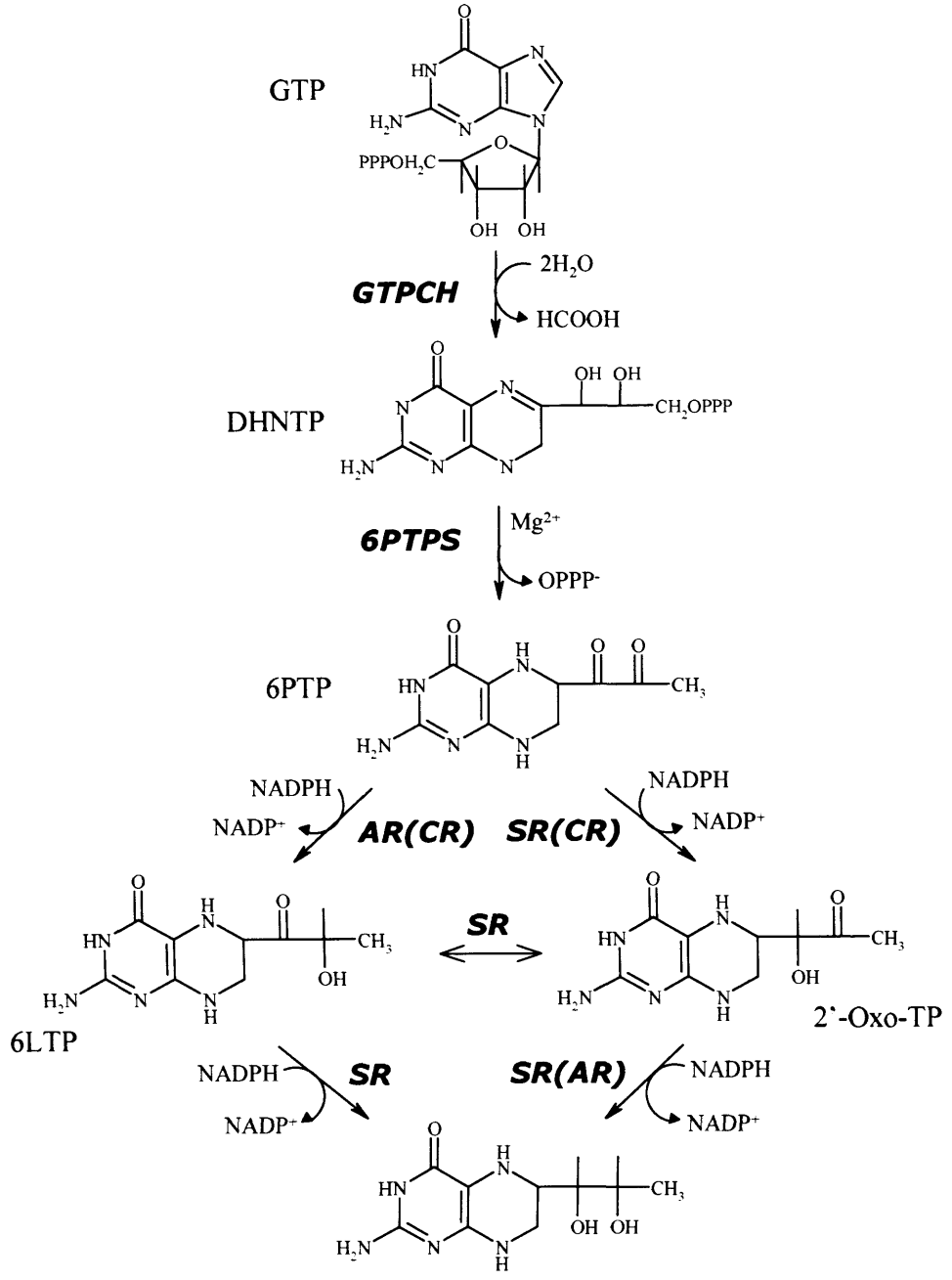
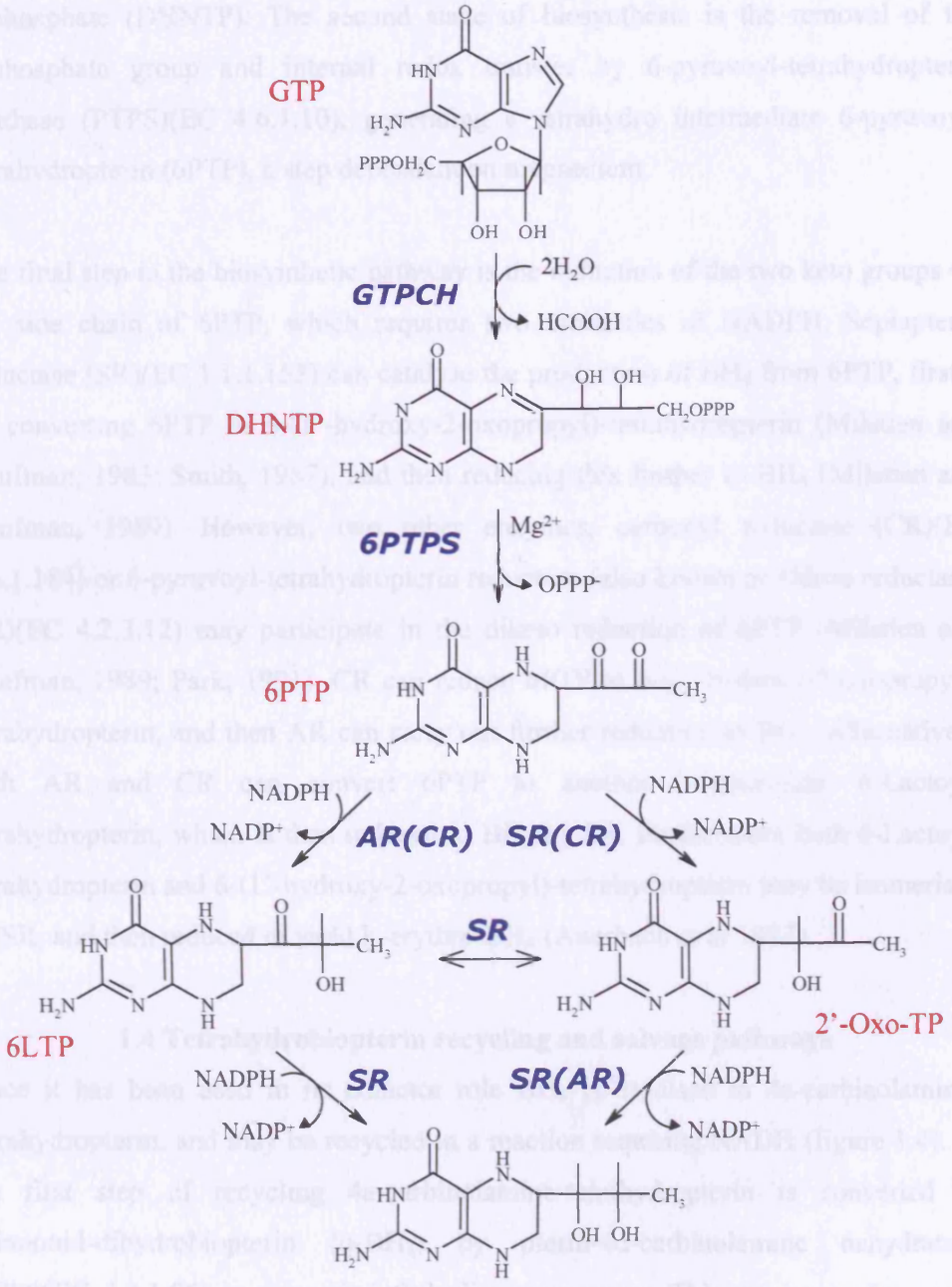


Figure 1.3. Biosynthetic pathway of BH_4.

Abbreviations: GTP; guanosine 5'-triphosphate; DHNTP, 7,8-Dihydroneopterin triphosphate; 6PTP, 6-Pyruvoyl-tetrahydropterin; 6LTP, 6-Lactoyl-tetrahydropterin; 2'-Oxo-TP, 6-(1'-hydroxy-2-oxopropyl)-tetrahydropterin; NADPH, β-nicotinamide adenine dinucleotide phosphate; GTP-CH, GTP cyclohydrolase I; 6PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase; AR, aldose reductase; CR, carbonyl reductase.



5,6,7,8-Tetrahydrobiopterin

Figure 1.3. Biosynthetic pathway of BH_4.

Abbreviations: GTP; guanosine 5'-triphosphate; DHNTP, 7,8-Dihydroneopterin triphosphate; 6PTP, 6-Pyruvoyl-tetrahydropterin; 6LTP, 6-Lactoyl-tetrahydropterin; 2'-Oxo-TP, 6-(1'-hydroxy-2-oxopropyl)-tetrahydropterin; NADPH, β-nicotinamide adenine dinucleotide phosphate; GTP-CH, GTP cyclohydrolase I; 6PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase; AR, aldose reductase; CR, carbonyl reductase.

triphosphate (DHNTP). The second stage of biosynthesis is the removal of the triphosphate group and internal redox transfer by 6-pyruvoyl-tetrahydropterin synthase (PTPS)(EC 4.6.1.10), generating a tetrahydro intermediate 6-pyruvoyl-tetrahydropterin (6PTP), a step dependent on magnesium.

The final step in the biosynthetic pathway is the reduction of the two keto groups on the side chain of 6PTP, which requires two molecules of NADPH. Sepiapterin reductase (SR)(EC 1.1.1.153) can catalyse the production of BH_4 from 6PTP, firstly by converting 6PTP to 6-(1'-hydroxy-2-oxopropyl)-tetrahydropterin (Milstien and Kaufman, 1983; Smith, 1987), and then reducing this further to BH_4 (Milstien and Kaufman, 1989). However, two other enzymes, carbonyl reductase (CR)(EC 1.1.1.184) or 6-pyruvoyl-tetrahydropterin reductase (also known as aldose reductase; AR)(EC 4.2.3.12) may participate in the diketo reduction of 6PTP (Milstien and Kaufman, 1989; Park, 1991). CR can reduce 6PTP to 6-(1'-hydroxy-2-oxopropyl)-tetrahydropterin, and then AR can carry out further reduction to BH_4. Alternatively both AR and CR can convert 6PTP to another intermediate, 6-Lactoyl-tetrahydropterin, which is then reduced to BH_4 by SR. Furthermore both 6-Lactoyl-tetrahydropterin and 6-(1'-hydroxy-2-oxopropyl)-tetrahydropterin may be isomerised by SR, and then reduced to yield L-erythro-BH_4 (Auerbach *et al* 1997).

1.4 Tetrahydrobiopterin recycling and salvage pathways

Once it has been used in its cofactor role BH_4 is oxidised to 4a-carbinolamine-tetrahydropterin, and may be recycled in a reaction requiring NADH (figure 1.4). In the first step of recycling 4a-carbinolamine-tetrahydropterin is converted to quinonoid-dihydrobiopterin (q-BH_2) by pterin-4α-carbinolamine dehydratase (PCD)(EC 4.2.1.96) in a reaction that eliminates water. This step may also occur non-enzymically, however not at a rate sufficient to keep BH_4 in a reduced state (Bailey *et al*, 1993). Dihydropteridine reductase (DHPR)(EC 1.6.99.7;) then converts q-BH_2 back to BH_4 in an NADH dependent step (Kaufman, 1964). This process is known as the recycling pathway. Linked to this is another set of reactions known as the salvage pathway, which can also regenerate BH_4. Because q-BH_2 is unstable, it can undergo non-enzymic conversion to the more stable 7,8-dihydrobiopterin (BH_2). BH_2 is then converted back to BH_4 by dihydrofolate reductase (DHFR)(EC 1.5.1.3)

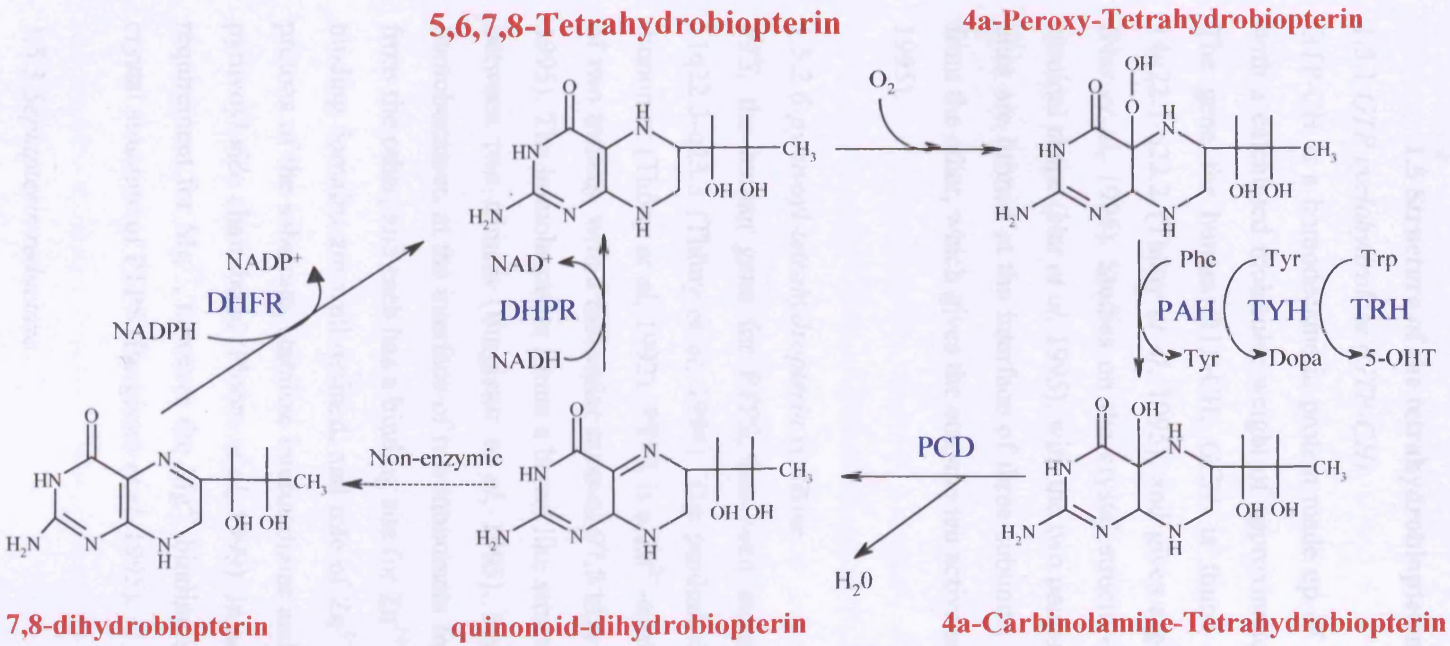


Figure 1.4. The tetrahydrobiopterin recycle and salvage pathways (adapted from Ponzone *et al*, 2004). Abbreviations; PAH, phenylalanine hydroxylase; TYH, tyrosine hydroxylase; TRH, tryptophan hydroxylase; PCD, pterin-4α-carbinolamine dehydratase; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase; NADH, β-nicotinamide adenine dinucleotide.

in an NADPH-dependent step (Kaufman, 1964). These pathways are shown in figure 1.4.

1.5 Structure of the tetrahydrobiopterin biosynthetic enzymes

1.5.1 *GTP cyclohydrolase (GTP-CH)*

GTP-CH is a homodecameric protein made up of two identical pentamer subunits, with a calculated molecular weight of approximately 279kDa (Togari *et al*, 1992). The gene for human GTP-CH, *GCH*, is found on chromosome 14 at position 14q22.1-q22.2 (Thöny *et al*, 1995), and gives a gene product of 221 amino acids (Nar *et al*, 1995). Studies on the crystal structure of GTP-CH show it to have a toroidal shape (Nar *et al*, 1995), with the two pentamers on top of each other. Active sites are formed at the interface of three subunits; two from one pentamer and one from the other, which gives the enzyme ten active sites per functional unit (Nar *et al*, 1995).

1.5.2 *6-pyruvoyl-tetrahydropterin synthase*

PTS, the human gene for PTPS, has been mapped to the chromosomal region 11q22.3-q23.3 (Thöny *et al*, 1994). The product of this gene is a 145 amino acid monomer (Thöny *et al*, 1992). PTPS is a Zn^{2+}-containing homohexamer, comprised of two trimers, with a molecular mass of 97.5 kDa (Nar *et al*, 1994; Burgisser *et al*, 1995). The homohexamer forms a barrel like structure, with face-to-face association between two trimers (Burgisser *et al*, 1995). Six active sites are formed in the homohexamer, at the interface of two monomers from one trimer, and one monomer from the other, and each has a binding site for Zn^{2+} (Burgisser *et al*, 1995). The Zn^{2+} binding domains are well defined, and role of Zn^{2+} in the reaction is to activate the protons of the substrate, stabilize intermediates and prevent breakage of the C1'C2' pyruvoyl side chain bond (Ploom *et al*, 1999). In addition to Zn^{2+}, PTPS has a strict requirement for Mg^{2+}, however the Mg^{2+} binding site has yet to be resolved in the crystal structure of PTPS (Burgisser *et al*, 1995).

1.5.3 *Sepiapterin reductase*

In humans, the gene for SR known as *SPR*, has been mapped to chromosomal region 2p14-2p12, which produces a protein of 261 amino acids (Thöny *et al*, 1995). SR is a homodimeric enzyme, with a predicted molecular mass of 56 kDa (Auerbach *et al*,

1997). The crystal structure of this enzyme shows that the two monomers are anti-parallel to one another at a 90° angle, when in their active conformation, and the substrate 6PTP and cofactor NADPH bind from separate sides of the enzyme (Auerbach *et al*, 1997).

1.6 Regulation of tetrahydrobiopterin homeostasis

The regulation of BH_4 concentration in cells appears to occur mainly through modulation of biosynthesis. Many different agents are able to alter BH_4 levels in mammals. Pro-inflammatory stimuli like the bacterial endotoxin lipopolysaccharide (LPS), cytokines such as interferon γ (IFN-γ), hormones including insulin, and other stimuli such as cyclic adenosine monophosphate (cAMP) and the neuroleptic reserpine (Werner *et al*, 1998) can all increase BH_4. The anti-inflammatory cytokine interleukin-4, drugs like dexamethasone, glucocorticoids and the neurohormone melatonin all decrease BH_4 levels in a number of cell types (Werner *et al*, 1998). These stimuli appear to have their effect mainly by influencing GTP-CH activity and expression, although modulation of PTPS and SR, the other enzymes in the pathway, occurs to some extent (Kerler *et al*, 1989; Mori *et al*, 1997; Franscini, 2003).

One mechanism by which GTP-CH activity can be modulated, is through GTP cyclohydrolase feedback regulatory protein (GFRP). Initially named "p35" (Harada *et al*, 1993), this 52 kDa homopentameric protein, with momomers consisting of 83 amino acids, was later renamed GFRP (Milstien *et al*, 1996; Yoneyama *et al*, 1998). At elevated levels of BH_4, GFRP mediates inhibitory feedback regulatory effects on GTP-CH, while at high levels of phenyalanine it stimulates GTP-CH activity (Harada *et al*, 1993). Two GFRP homopentamers sandwich the homodecameric enzyme to exert the inhibitory and stimulatory actions (Maita *et al*, 2002). BH_4 and phenylalanine bind between the interface of the enzyme and GFRP, with the BH_4 binding pocket formed mainly by GTP-CH, and the phenylalanine binding site by GFRP. It is thought that conformational changes mediated by GFRP on GTP-CH are responsible for changes in activity during feedback regulation (Maita *et al*, 2004).

In addition to BH_4 and phenylalanine, a number of other stimuli can influence GFRP, leading to altered BH_4 biosynthesis. Hydrogen peroxide and LPS down-regulate GFRP and increase GTP-CH mRNA, leading to an increase in BH_4 (Werner *et al*,

2002; Gesierich *et al*, 2003; Ishii *et al*, 2005; Kalivendi *et al*, 2005). Furthermore, 2,4-diaminohydroxypyrimidine (DAHP), an inhibitor of GTP-CH, exerts its effect through engaging GFRP (Xie *et al*, 1998; Kolinsky and Gross, 2004). GFRP mRNA has been detected throughout human tissues, and in high quantities in human liver, kidney, heart, lung, skin and brain (Kapatos *et al*, 1999; Gesierich *et al*, 2003; Chavan *et al*, 2006). It is expressed in serotonergic neurones, where it modulates the levels of BH_4, but is undetectable in dopaminergic cells (Kapatos *et al*, 1999). Therefore GFRP would appear to have a widespread functional role throughout the periphery and CNS, although its precise physiological role in the brain remains unclear.

GTP-CH function can also be regulated by phosphorylation. GTP-CH has several putative phosphorylation sites for protein kinase C (EC 2.7.1.37), which may increase activity of the enzyme (Hatekeyama *et al*, 1991; Imazumi *et al*, 1994; Hesslinger *et al*, 1998). Regulation of GTP-CH may also occur at the level of mRNA synthesis, as cAMP has been demonstrated to prolong the half-life of GTP-CH mRNA, leading to increased BH_4 (Plüss *et al*, 1999). Activity of GTP-CH is also modulated by the concentration of substrate GTP, which binds cooperatively to GTP-CH, thus altering the enzyme kinetics (Thöny *et al*, 2000). In addition, *GCH* splice variants may have a role in modulating GTP-CH expression, by reducing the amount of wild type enzyme present (Pandya *et al*, 2006).

PTPS may also play a regulatory role in BH_4 biosynthesis. In murine neuroblastoma cell lines, both GTP-CH and PTPS expression increase in response to LPS (Mori *et al*, 1997). In human endothelial cells PTPS expression is induced 4-fold by Interleukin-β (IL-β), however this accompanies a 300-fold increase in GTP-CH expression, which suggests that in human cells under immune stimulation, PTPS may become the rate limiting step (Franscini *et al*, 2003). When PTPS is limiting in humans, a build up of dihydroneopterin and neopterin, the stable breakdown products of DHNTP occurs, due to the high amount of substrate relative to PTPS. Indeed, the build up of neopterin is often used as a marker of immune system activation in humans (Werner *et al*, 1991). PTPS may also be phosphorylated in humans, at serine-19 by cGMP-dependent protein kinases, which leads to 3-fold greater activity (Scherer-Oppliger *et al*, 1999). Furthermore, a patient with a mutation in the

phosphorylation domain of PTPS was shown to be BH_4-deficient, suggesting that phosphorylation of PTPS is important for normal BH_4 metabolism (Oppliger *et al*, 1995).

SR may also play an important role in regulating BH_4 biosynthesis. Like GTP-CH and PTPS, expression and activity of SR is increased following cytokine and bacterial endotoxin stimulation (Ziegler *et al*, 1990; Mori *et al*, 1997), but not to the same extent as GTP-CH. Phosphorylation of SR also occurs via protein kinase C (EC 2.7.1.37) or calcium/calmodulin protein kinase II (EC 2.7.1.123)(Katoh *et al*, 1994; Fujimoto *et al*, 2002), and appears to increase activity of the enzyme. However, work so far has only been done in recombinant purified enzyme, so it remains to be demonstrated whether phosphorylation of SR effects activity in intact cells. There is also some evidence of feedback inhibition on SR activity, by catecholamines noradrenaline (NA) and serotonin (5-HT), and by the indolamines melatonin and N-acetyl-serotonin in rat brain homogenates (Katoh *et al*, 1982). N-acetyl-serotonin is a potent specific inhibitor of SR activity (Smith *et al*, 1992)

It has also been proposed that alterations in DHPR activity may also impact on BH_4 homeostasis. Metals such as aluminium, cadmium, mercury, lead and manganese may inhibit DHPR *in vitro* (Altindag *et al*, 2003). In addition, there are correlations between serum concentrations of lead and BH_4, and intelligence quotients in humans (Blair *et al*, 1982). Furthermore, patients on haemodialysis were found to have increased serum levels of aluminium, that are associated with decreased DHPR activity and BH_4 levels (Altmann *et al*, 1987). However, these findings are controversial, as contrasting data that did not find any association between aluminium, DHPR activity and cognitive function have been reported (Bolla *et al*, 1991).

1.7 Functions of Tetrahydrobiopterin

BH_4 is an essential cofactor for a number of enzymes including the aromatic amino acid mono-oxygenases, glyceryl ether mono-oxygenase, as well as all forms of nitric oxide synthase. BH_4 also appears to have additional roles not related to its cofactor function, all of which are discussed below.

1.7.1 *Aromatic amino acid mono-oxygenases*

BH_4 has a well-characterised role as a cofactor for the aromatic amino acid mono-oxygenases. Enzymes that use BH_4 as a cofactor in this group are phenylalanine hydroxylase (PAH)(EC 1.14.16.1), tyrosine hydroxylase (TH)(EC2.7.1.124) and tryptophan hydroxylase (TRH)(EC 1.14.16.4). PAH converts phenylalanine to tyrosine mainly in the liver, whilst TH then converts tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) in the central and peripheral nervous system. TRH converts tryptophan to 5-hydroxytryptophan, which is expressed widely in mammalian tissues (figure 1.5). Hepatic PAH is essential in maintaining peripheral phenylalanine homeostasis, as well as providing TH with substrate to generate L-dopa, which is essential in dopamine (DA), and subsequently adrenaline and NA biosynthesis. TRH provides 5-hydroxytryptophan, the precursor for 5-HT and melatonin.

Little is known about the precise reaction mechanisms of the aromatic amino acid mono-oxygenases, however they are thought to be very similar (Fitzpatrick, 1999). All require Fe^{3+}, O_2, and BH_4, and have a catalytic core that is highly conserved (Hufton *et al*, 1995; Grenett *et al*, 1987). BH_4 is thought to play a role as an electron donor in these enzymes, providing one electron to Fe^{3+} in the active site, and another to reduce molecular oxygen (Hufton *et al*, 1995). The activation of oxygen by BH_4 is thought to involve the formation of a 4a-peroxy-tetrahydrobiopterin intermediate (Fitzpatrick, 1999). Then following catalysis, a 4a-carbinolamine-tetrahydrobiopterin intermediate is generated (Wei *et al*, 2003)(figure 1.4). The 4a-peroxo-pterin intermediate is thought to be the hydroxylating species in the reaction, however this is still a matter of debate (Wei *et al*, 2003).

Once hydroxylation is complete, 4a-carbinolamine-tetrahydrobiopterin is then converted back to BH_4. This occurs firstly via pterin 4a-carbinolamine dehydratase (PCD)(EC 4.2.1.96), which produces q-BH_2. q-BH_2 is then reduced to BH_4 by DHPR in a step requiring NADH, ready for another round of catalysis (figure 1.4). PAH may form a complex with both PCD and DHPR to enable more efficient catalysis (Citron *et al*, 1992).

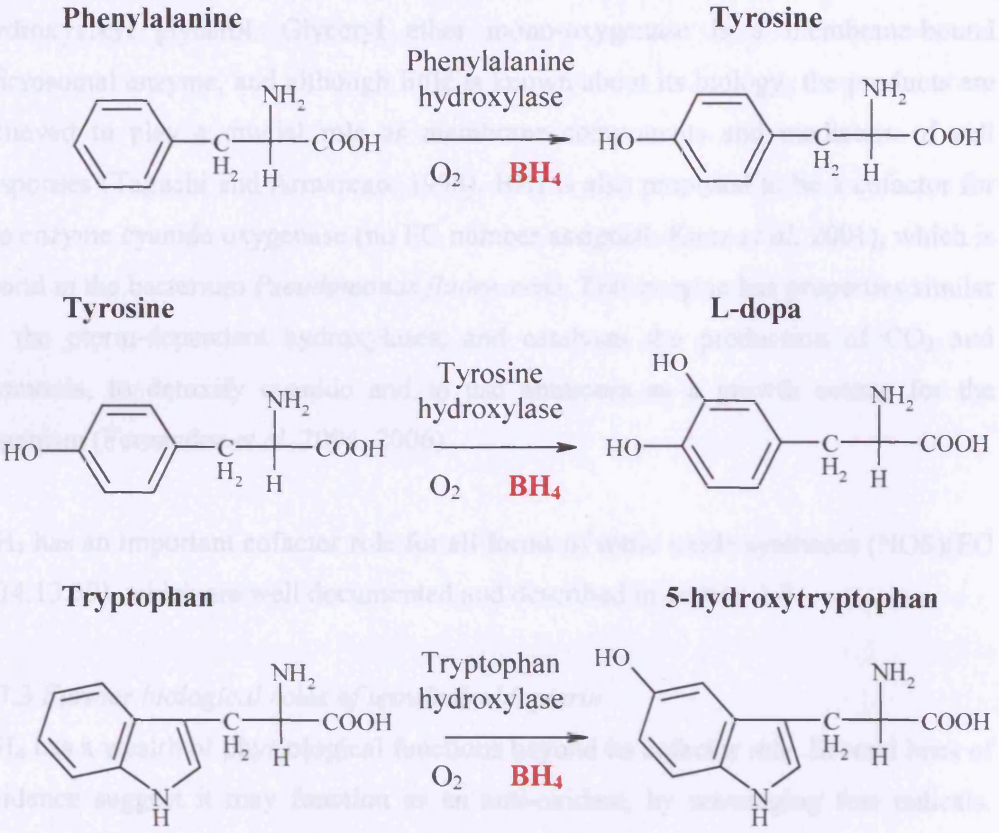


Figure 1.5. Reactions catalysed by BH_4-dependent aromatic amino acid mono-oxygenases

1.7.2 *Further cofactor roles of tetrahydrobiopterin*

As well as the aromatic amino acid mono-oxygenases, BH_4 is also required as a cofactor for glyceryl ether mono-oxygenase (EC 1.14.16.5)(Tietz *et al*, 1964). This enzyme hydroxylates the α-carbon of the lipid chain in glyceryl ether, producing α-hydroxyalkyl glycerol. Glyceryl ether mono-oxygenase is a membrane-bound microsomal enzyme, and although little is known about its biology, the products are believed to play a crucial role as membrane components and mediators of cell responses (Taguchi and Armarego, 1998). BH_4 is also proposed to be a cofactor for the enzyme cyanide oxygenase (no EC number assigned; Kunz *et al*, 2001), which is found in the bacterium *Pseudomonas fluorescens*. This enzyme has properties similar to the pterin-dependent hydroxylases, and catalyses the production of CO_2 and ammonia, to detoxify cyanide and to use ammonia as a growth source for the organism (Fernandez *et al*, 2004, 2006).

BH_4 has an important cofactor role for all forms of nitric oxide synthases (NOS)(EC 1.14.13.39), which are well documented and described in section 1.8.

1.7.3 *Further biological roles of tetrahydrobiopterin*

BH_4 has a wealth of physiological functions beyond its cofactor role. Several lines of evidence suggest it may function as an anti-oxidant, by scavenging free radicals. BH_4, dihydrobiopterin, as well as dihydroneopterin, are all readily oxidised by reactive oxygen species (ROS) *in vitro* (Heales *et al*, 1988; Milstien and Katusic, 1999), and BH_4 can protect cultured rat hepatocytes against superoxide-induced cell toxicity (Kojima *et al*, 1995). Other instances of BH_4 having a protective role in cells and *ex vivo* tissue include in dopaminergic neurones, where BH_4 may substitute for the antioxidant glutathione (GSH)(Nakamura *et al*, 2000), and in organotypic nigral slices where it may protect against 1-methyl-4-phenylpyridinium (MPP^+) induced oxidative stress (Madsen *et al*, 2003). BH_4 may protect against NO-toxicity, both through scavenging NO as well as being a cofactor for NOS. Therefore it is implicated in diseases where NO toxicity may be part of the pathogenesis, for instance in cardiovascular disease (Werner *et al*, 2003; Alp and Channon, 2003). However, other literature suggests that BH_4 can be damaging to cells under certain conditions. For instance, BH_4 increases in ischaemia (Cho *et al*, 1999; Kidd *et al*,

2005), and mediates dopaminergic cell death in rats treated with MPP^+ (Choi *et al*, 2003). The protective/toxic effects of BH_4 are further discussed in section 1.11.7.

BH_4 may also function in cells via a signalling and proliferative capacity independent of its cofactor role. Brain microdialysis studies in the rat brain show that BH_4 can stimulate release of DA, 5-HT and glutamate (Mataga *et al*, 1991) by activating neuronal Ca^{2+} channels (Koshimura *et al*, 2000). BH_4 can also stimulate release of L-Dopa from striatal tissue by a mechanism involving NO or free radicals derived from NO (Abreu-Gonzalez *et al*, 2006). Furthermore, BH_4 increases the survival of the dopaminergic PC12 cell line following serum and growth factor removal (Koshimura *et al*, 2000), and has a proliferative action on haemopoietic cells (Tanaka *et al*, 1989), as well as on human and mouse erythroleukaemia cell lines (Kerler *et al*, 1990; Zhuo *et al*, 1996).

1.8 Nitric oxide synthase

BH_4 is a cofactor for NOS (EC 1.14.13.39). Three isoforms of NOS are well documented to date; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). These enzymes convert L-arginine to L-citrulline, a reaction that requires O_2 and generates the free radical nitric oxide (NO)(figure 1.6).

1.8.1 *General structure and function of nitric oxide synthases*

Although the isoforms of NOS derive from separate genes, they all have essentially the same basic structure and carry out the same reaction, suggesting a common ancestral gene (Alderton *et al*, 2001). They are homodimers with calcium/calmodulin linker sequences, which contain the tightly bound cofactors BH_4, NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphryrin IX (haem)(Griffith and Stuehr, 1995). Each subunit contains N-terminal oxygenase, and C-terminal reductase domains, and catalyses the reaction of L-arginine, NADPH and O_2 to citrulline, $NADP^+$ and NO (figure 1.6). The reductase domain of NOS contains the flavins NADPH, FAD and FMN, and is involved in the transfer of electrons from NADPH, to reduce the iron in the haem group, which is part of the oxygenase domain. The oxygenase domain contains the substrate-binding pocket, BH_4 binding domain, as well as the aforementioned haem group, and performs the

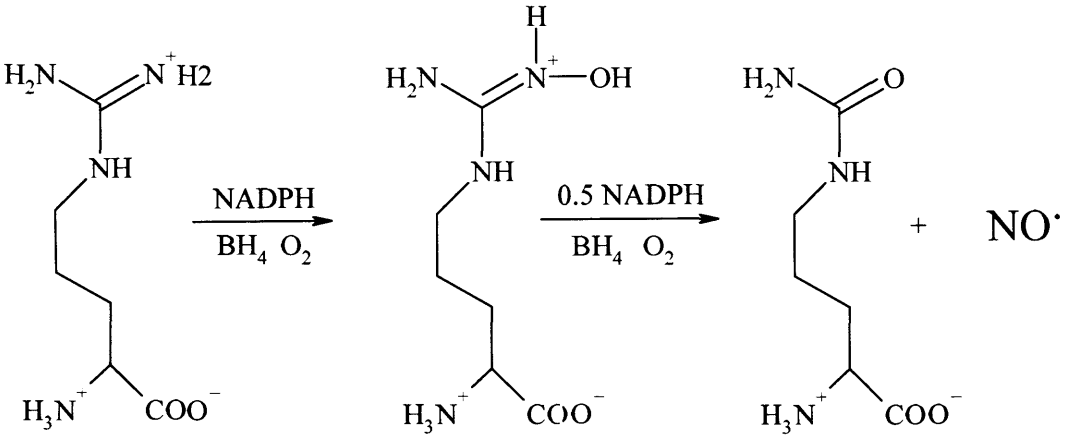


Figure 1.6. Reaction scheme for the conversion of L-Arginine to L-Citrulline, generating Nitric oxide (NO). N-^{G} Hydroxy-L-arginine is an intermediate in this reaction.

(Adapted from Stuehr *et al*, 1991)

two-step oxidation of arginine to citrulline. This domain structure is similar to the cytochrome P450 mono-oxygenases (EC 1.6.2.4)(Narhi and Fulco, 1987; Stuehr and Ikeda-Saito, 1992), which also transfer electrons from NADPH via FAD and FMN to haem, however NOS is unique in the requirement for the BH_4 cofactor and calmodulin regulation. Once the iron in the haem group is reduced, it is suggested that this species activates molecular oxygen for substrate activation, and generates water (Adak *et al*, 2000).

NOS is only active in producing NO in its dimer form. Dimerisation is thought to occur between oxygenase domains on the monomers, at the BH_4 binding sites, and involves BH_4, haem and substrate (Crane *et al*, 1998). Dimerisation may assist the transfer of electrons from the oxygenase to reductase domains (Siddhanta *et al*, 1996), which suggests one reason why NOS monomers are not active. In addition L-arginine fails to bind in the absence of a dimer (Crane *et al*, 1997), suggesting that dimer formation plays a role in regulating enzyme activity (Li and Poulos, 2005). NOS also contains Zn^{2+}, which is also thought to play an important role for dimerisation, but is not essential for catalysis (Miller *et al*, 1999).

1.8.2 *Biosynthesis of nitric oxide*

The synthesis of NO is a two-step process that is coupled to the five-electron oxidation of L-arginine to citrulline (figure 1.7). The products citrulline and NO are formed at a 1:1 stoichiometry, along with two molecules of water (Stuehr *et al*, 1991a). The first step in the reaction is the generation of an intermediate N-^{G} Hydroxy-L-arginine (Stuehr *et al*, 1991b). This occurs when two electrons from NADPH are transferred via FAD then FMN in the reductase domain, to the Fe^{3+} in the haem complex of the oxygenase domain, which enables the activation of molecular oxygen, and leads to the monooxygenation of the guanidine nitrogen in L-arginine (Hurshman *et al*, 1999, 2003; Li and Poulos, 2005). N-^{G} Hydroxy-L-arginine is the product of this pathway, which consumes 1 mole of NADPH and 1 mole of O_2 (Stuehr *et al*, 1991b). The second step of catalysis is less clear, but requires a three-electron monooxygenation of N-^{G} Hydroxy-L-arginine. This reaction uses 0.5 moles of NADPH, and 1 mole of O_2, and a vital role for BH_4 is suggested which is discussed below (Adak *et al*, 2000).

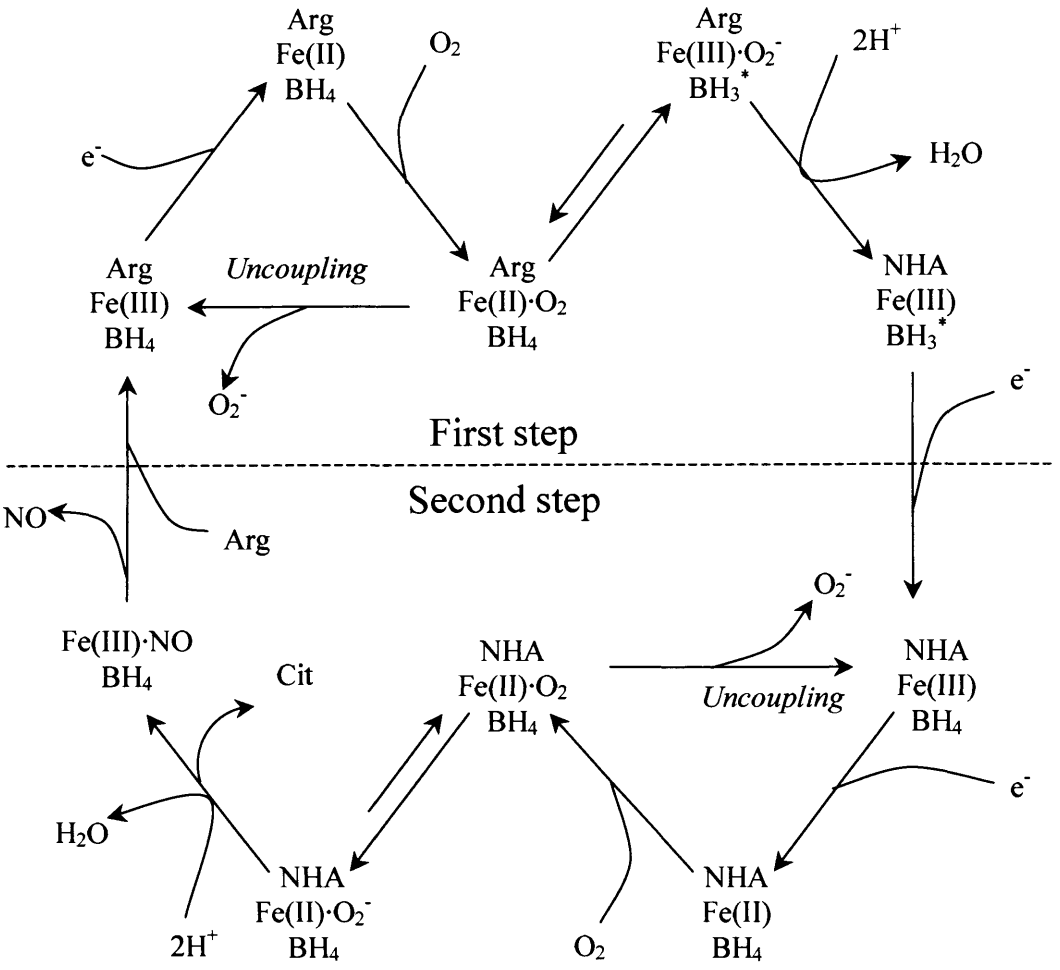


Figure 1.7. The proposed role of BH_4 in NO generation. BH_4 is thought to act as a one electron donor in both stages of catalysis. This generates a BH_3* radical. In the absence of Arginine or BH_4 the reaction is uncoupled and superoxide ($\cdot O_2^-$) is generated.

Abbreviations: Arg, Arginine; Cit, Citrulline; NHA, N-^{G} Hydroxy-L-arginine.

(Adapted from Werner *et al*, 2003).

1.8.3 *Role of tetrahydrobiopterin in nitric oxide synthesis*

The role of BH_4 in NO synthesis is controversial, and so far a number of different theories exist as to its function (Alderton *et al*, 2001). It was initially suggested that BH_4 functions as it does in the aromatic amino acid mono-oxygenases (Tayeh and Marletta, 1989), however it was subsequently discovered that BH_4 remains tightly bound to the enzyme during and after catalysis, and is not recycled by the salvage or recycling pathways (Giovanelli *et al*, 1991). Furthermore there are discrepancies with the amount of product formed per mole of BH_4. In the aromatic amino acid mono-oxygenases stoichiometric amounts of product are formed with 1 mole of BH_4, whereas 15 moles of citrulline are generated for every mole of BH_4 (Giovanelli *et al*, 1991). In addition the K_m of BH_4 for NOS is typically 0.02μM (Giovanelli *et al*, 1991), in contrast to 2μM for PAH (Abita *et al*, 1984). It now appears that BH_4 may be a one electron donor during catalysis, reducing the oxyferrous haem, which results in the generation of a BH_3* radical (Hurshman *et al*, 1999). The scheme for these sets of reactions is illustrated in figure 1.7 (Werner *et al*, 2003).

BH_4 appears to be necessary for both stages of NOS catalysis to occur, suggesting that BH_4 has a redox role in this reaction (Klatt *et al*, 1993; Gorren *et al*, 2005). In the first stage of catalysis BH_4 is proposed to donate an electron to $Fe^{2+}{\cdot}O_2$ arginine complex, which leads to oxygen activation and hydroxylation of arginine to N-^{G} Hydroxy-L-arginine (Werner *et al*, 2003). This step creates the BH_3* radical, and induces a shift in the NOS haem from low- to high-spin, which aids enzyme activation (Gorren and Mayer, 2002). It is proposed that the BH_3* radical then remains bound to NOS and is reduced back to BH_4 by an electron from the reductase domain (Wei *et al*, 2003). This would allow it to participate in a further round of NO synthesis and may explain why a recycling and salvage pathway is not found for this enzyme (Giovanelli *et al*, 1991).

In addition to catalysis, BH_4 plays a well-documented role as an allosteric regulator of NOS, in the dimerisation of the enzyme and substrate binding. BH_4 may also control the steady state level of NOS. The importance of dimerisation for full catalytic activity, along with the role of BH_4, was first shown in 1997 (Baek *et al*, 1993). The NOS homodimer is formed at the interface of the BH_4 binding site (Crane *et al*, 1998). BH_4, arginine and haem incorporation are all important for NO

generation, however they are not essential for dimerisation in every isoform of NOS (Venema *et al*, 1997). But for dimers to become stable and active enzyme structures, BH_4 and arginine binding are necessary (Baek *et al*, 1993; Ghosh *et al*, 1997). BH_4 is known to increase the affinity of the enzyme for arginine, and vice versa, which is also crucial for NOS activity (Klatt *et al*, 1994). Increasing the concentration of BH_4 in nNOS enzyme assays lowers the K_m for arginine (Gorren *et al* 1996), whereas decreasing BH_4 concentrations by approximately 50% increases the K_m from 9.5μM to 53.3μM (Brand *et al*, 1995). Loss of BH_4 may be an endogenous signal for ubiquitylation and degradation of nNOS (Kamada *et al*, 2005). This can lead to a rapid decrease in the steady state concentrations of nNOS that is not readily reversed.

In the presence of low concentrations of BH_4 and arginine, NOS catalyses the formation of superoxide ($\cdot O_2^-$) (Heinzel *et al*, 1992; Pou *et al*, 1992). This is thought to occur because BH_4 is no longer present to transfer an electron to the $Fe^{2+}\cdot O_2$, thus hydroxylation of arginine does not occur, and the reaction becomes uncoupled generating $\cdot O_2^-$ (Rusche *et al*, 1998). This effect is observed *in vitro* in purified enzyme extracts (Xia *et al*, 1998; Rosen *et al*, 2002) and in endothelial cells, where greater amounts of $\cdot O_2^-$ were observed with declining BH_4 levels (Kuzkaya *et al*, 2003). This uncoupled reaction is proposed to be part of the pathophysiology of various disorders related to oxidative stress, which are discussed in section 1.11.6.

1.9 Nitric oxide synthase isoforms

1.9.1 *Neuronal nitric oxide synthase*

Neuronal NOS (nNOS), also known as NOS 1 or type 1 NOS, is found on chromosome 12 at position 12q24.2 in humans (Kishimoto *et al*, 1992). It is a Ca^{2+}/calmodulin-dependent constitutively expressed isoform, which was initially found in neurones but has subsequently been localised in the periphery, such as in skeletal muscle (Nakane *et al*, 1993), kidney (Jarry *et al*, 2003) and has a well publicised role in the penis (Magee *et al*, 1996). nNOS is a protein of approximately 161 kDa, and is made up of 1434 amino acids (Hall *et al*, 1994). It is expressed in the cytosol, and is active only as a homodimer, producing picomolar quantities of NO (Geller and Billiar, 1998). In addition this enzyme contains an N-terminal 220 amino acid region that is specific to nNOS and may be a site for membrane attachment (Brenman *et al*, 1996). In the brain nNOS is widely expressed, prominently in

neurones in the cerebellum and hippocampus, as well as in the cortex and striatum (Wolf, 1997).

1.9.2 *Inducible nitric oxide synthase*

Inducible NOS (iNOS), is also referred to as NOS II and type II NOS. The human gene for iNOS is located on chromosome 17 at 17q11-q12 (Marsden *et al*, 1994), and encodes a protein of 1153 amino acids with a molecular mass of 131 kDa (Geller *et al*, 1993). Typically it is not constitutively expressed in most cell types, although "constitutive" expression has been demonstrated in certain tissues, such as the mouse ileal mucosa (Hoffman *et al*, 1997). It is independent of calcium for activity, which may be because it is very tightly bound to calmodulin, due to the presence of a binding sequence on this isoform (Cho *et al*, 1992). Inducible NOS is widely expressed throughout mammalian tissues, including in the brain (Geller and Billiar, 1998), and is expressed in response to the presence of cytokines such as IFN-γ, as well as components of bacterial membrane, such as LPS, and even viruses (Gross and Wolin, 1995). This, coupled with the high concentration (micromolar) of NO that iNOS generates (Geller and Billiar, 1998), has lead to suggestions that iNOS may have a role in immune response.

1.9.3 *Endothelial nitric oxide synthase*

Endothelial NOS (eNOS) is also known as NOS III or type III NOS. It is a protein of 1203 amino acids with a predicted molecular mass of 133 kDa (Janssens *et al*, 1992) and in humans is localised to chromosome 7, position 7q35-q36 (Marsden *et al*, 1993). Endothelial NOS is a constitutive calcium/calmodulin-dependent isoform, which produces picomolar quantities of NO (Geller and Billiar, 1998). It is found in endothelial cells where it was first identified (Pollock *et al*, 1991), however it has subsequently been located in a wide variety of other cell types, and is expressed in the brain (Li *et al*, 2002). In the endothelium eNOS is localised to the caveolae, the microdomains of the plasma membrane involved in signal transduction (Shaul *et al*, 1996). Endothelial NOS can be activated by shear stress caused by blood flow through the endothelium (Shaul *et al*, 2002), and plays a role in maintaining vascular tone (Vallance *et al*, 1989), thus it is regarded as having an important function throughout the cardiovascular system.

1.9.4 *Mitochondrial nitric oxide synthase*

There are suggestions there is an isoform of NOS associated with mitochondria (mtNOS), a finding that is still controversial. This discovery was based on immunocytochemical evidence, showing NOS antibody binding to mitochondrial membranes in rat brain, liver, heart, skeletal muscle and kidney, and it was proposed that this form of NOS represents a novel regulatory pathway of energy metabolism (Bates *et al*, 1995, 1996; Elfering *et al*, 2002). The molecular mass of mtNOS is proposed to be approximately 130 kDa (Tatoyen and Giulivi, 1998) and appears to have some functional similarities with nNOS. It is also absent in nNOS knockout mice (Kanai *et al*, 2001), which lead to suggestions that it is a splice variant of nNOS. However, studies looking at the cross reactivity of antibodies for the different isoforms of NOS demonstrated that mtNOS reacted with iNOS, but not nNOS or eNOS antibodies (Tatoyen and Giulivi, 1998). It is still unproven whether mtNOS is specifically associated with mitochondria and not associated with other organelles that are isolated with the mitochondrial fraction, such as lysosomes. This has lead to suggestions that mtNOS is a membrane-associated form of iNOS (Alderton *et al*, 2001), which is consistent with earlier reports of iNOS in macrophages (Hiki *et al*, 1991). Whether mtNOS exists or not, it has been proposed to play a role in cardiovascular physiology (Fellet *et al*, 2006) and learning and memory (Lores-Arnaiz *et al*, 2006).

1.10 Biological role of nitric oxide

Nitric oxide was first discovered as a biological mediator in 1980, via experiments investigating how acetylcholine mediated its vasodilatory effects on smooth muscle cells (Furchgott and Zawadzki, 1980). Initially it was thought acetylcholine acted directly on these cells causing dilatation, however it was observed that a small molecule mediated these effects by acting via specific receptors on endothelial cells. This molecule was initially termed endothelial derived relaxing factor (EDRF). Separate studies examining how nitro-glycerine mediates its vasodilatory effects in treating angina, demonstrated that NO was the active molecule, and that NO causes dilatation by activating guanylate cyclase leading to cyclic guanosine 3',5'-monophosphate (cGMP) formation (Arnold *et al*, 1977; Ignarro *et al*, 1981). These observations lead to the suggestion that EDRF and NO are the same molecule, which was confirmed in 1987 (Palmer *et al*, 1987).

Since the elucidation of a role for NO in maintaining vascular tone, a great wealth of literature has emerged on the diverse biological functions of NO, some of which are discussed below. NO exerts many of these actions via activation of guanylate cyclase and formation of cGMP. NO interacts with haem-iron prosthetic groups on guanylate cyclase (Ignarro *et al*, 1982), causing activation of the enzyme, converting GTP to cGMP, which then engages various downstream targets such as phosphodiesterases and ion channels (Blaise *et al*, 2005). In addition to its role in smooth muscle tone discussed above, NO mediates other biological responses via cGMP, such as inhibiting platelet aggregation and blood clotting (Radomski *et al*, 1990), as well as a role in penile erection (Pickard *et al*, 1995; Magee *et al*, 1996; Sommer *et al*, 2006).

Another important function of NO is in the immune response to invading pathogens. Inducible NOS expression, and NO generation are activated several fold by cytokines and by LPS, both *in vitro* and *in vivo* (Werner *et al*, 1993; Bolaños *et al*, 1994; Bune *et al*, 1996). Inducible NOS is capable of generating micromolar levels of NO, and can sustain this production for many hours (Geller and Billiar, 1998), which gives this enzyme antimicrobial properties and is even known to inhibit the growth of viruses (Karupiah *et al*, 1993; Sakai *et al*, 2006). The induction of iNOS is also accompanied by a parallel induction of GTP-CH expression (Hattori and Gross, 1993), presumably to provide sufficient BH_4 to act as cofactor for iNOS. Due to the highly diffusible nature of NO, it can freely move from the cell where it is produced, to the target cell to cause intracellular damage. This damage may occur though direct chemical reaction with intracellular proteins. S-Nitrosylation is the attachment of NO to the thiol group of a cysteine residue, and can inhibit many enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(EC 1.2.1.13)(Padgett and Whorton, 1995), protein kinase C (EC 2.7.1.37)(Gopalakrishna *et al*, 1993), glutathione reductase (EC 1.8.1.7)(Becker *et al*, 1995), aconitase in the citric acid cycle (EC 4.2.1.3)(Hibbs *et al*, 1988), cytochrome c oxidase (complex IV; EC 1.9.3.1) and succinate cytochrome c reductase (complex II-III; EC 1.8.3.1) of the mitochondrial electron transport chain (Bolaños *et al*, 1994). Inhibition of these enzymes can result in toxic effects. In addition to its effects on enzyme activity, NO can mediate DNA strand breaks (Nguyen *et al*, 1992) and give rise to DNA mutations (Wink *et al*, 1991).

In the CNS all cells have the ability to synthesize NO (Werner *et al*, 2003). Initially, development of the CNS is dependent on NOS activity (Nachmany *et al*, 2006). Furthermore, extensive expression of the different NOS isoforms, coupled with the fact the brain has the highest expression of NOS of any organ so far examined (Salter *et al*, 1991), means that NO may have a very widespread role in CNS function. Because NO can diffuse so easily, it may modulate pre- as well as post-synaptic transmission, and the short half-life of NO means the signal is terminated rapidly (Garthwaite, 1991). In neurones, nNOS-derived NO may serve as a signalling molecule by activating glutamate receptors, in particular N-methyl-D-aspartate (NMDA) receptors (Garthwaite *et al*, 1988) to modulate neurotransmission. Neuronal NOS activity is stimulated by an influx of Ca^{2+} through NMDA receptors, which leads to a rise in NO. This in turn can modulate the NMDA receptor, and attenuate or potentiate the glutamatergic signal (Kiss and Vizi, 2001). This modulation of pre- and post-synaptic NMDA receptors has an effect on synaptic plasticity, and may be involved in long-term potentiation (LTP) and depression (LTD), which are proposed mechanisms for learning and memory (Garthwaite and Boulton, 1995).

In addition, NO may have a damaging role in various disorders of the periphery and CNS, some of which are discussed in section 1.11.6.

1.11 Disorders of tetrahydrobiopterin metabolism

BH_4 deficiencies are a heterogeneous group of disorders, with wide-ranging clinical as well as biochemical characteristics. Deficiencies of GTP-CH, PTPS, SR, PCD, and DHPR have all been reported and characterised, and now thanks to progress in diagnosis and treatment, most are no longer lethal. Understanding of this group of diseases began in 1969, through the description of two siblings with a "genetic variant of phenylketonuria" (Tada *et al*, 1970). These patients were later diagnosed as being DHPR deficient (Tada *et al*, 1980). Then in 1974, three patients were described as having phenylketonuria (PKU), who although diagnosed early and given a low phenylalanine diet, did not respond to treatment and died at an early age (Smith, 1974). In a similar case with a patient unresponsive to dietary treatment, a liver biopsy revealed normal PAH activity, which lead to the speculation that this syndrome was due to a defect in BH_4 metabolism (Bartholomé, 1974).

In classical PKU, mutations in PAH lead to impairment of phenylalanine catabolism, which elevates phenylalanine throughout the periphery and CNS, and may cause mental retardation and other neurological symptoms in patients. Phenylalanine can compete with other large neutral amino acids, such as tyrosine and tryptophan, for transporters in the blood brain barrier, leading to deficiencies of DA and 5-HT in the brain (Tourian and Sidbury, 1983; Hommes and Lee, 1990). Therefore, maintaining PKU patients on a low phenylalanine diet leads to normal neurological development (Güttler, 1984). However, in BH_4 deficiencies, although a low phenylalanine diet may correct the hyperphenylalaninaemia (HPA), neurotransmitter deficiencies still persist and neurological development is impaired. In 1974, Smith reasoned that a defect in BH_4 metabolism could lead to deficiencies of DA, 5-HT, adrenaline and NA in the brain, which could account for the persistent neurological abnormalities in these patients (Smith, 1974).

In the years immediately following this, a number of cases of HPA associated with BH_4 deficiency were described (Danks *et al*, 1975; Kaufman *et al*, 1975, 1978). This lead to the suggestion that pterins may be used in treating patients with BH_4 deficiencies, and Danks *et al* in 1975 demonstrated that it was possible to reduce blood phenylalanine levels in a patient with BH_4 deficiency, by administering synthetic BH_4 (Danks *et al*, 1975). Furthermore, it was also shown that therapy with L-dopa, carbidopa and 5-hydroxytryptophan, alone or combined with BH_4, can improve treatment for these patients (Bartholomé *et al*, 1975; Endres *et al*, 1982; McInnes *et al*, 1984).

Since these pioneering discoveries, a host of BH_4 deficiencies have been uncovered, which are discussed below. Inborn errors of BH_4 metabolism are largely autosomal recessive mutations in the enzymes that are involved in the biosynthesis or recycling of the cofactor. They make up approximately 1 to 2% of cases of HPA (Blau *et al*, 2001), and at the time of writing 534 cases of BH_4 deficiencies have been described in the International Database of Tetrahydrobiopterin Deficiencies (BIODEF) (Blau and Dhondt, 1998; http://www.bh4.org/BH4DatabasesBiodef.asp), with 193 different mutations described in these individuals (Thöny and Blau, 2006)(BIOMDB; International Database of Mutations Causing Tetrahydrobiopterin Deficiencies http://www.bh4.org/BH4_databases_biomdb.asp, Blau and Thöny, 1998). The

severity of these disorders varies depending on the enzyme mutation, however symptomatically they have much in common. Mental retardation, hypersalivation, convulsions, disturbances of posture and tone, lethargy, irritability, and temperature instability are some of the symptoms (Blau *et al*, 2001).

Screening tests for these individuals have been set up, which are carried out in infancy after detection of a prolonged HPA. It is suggested that screening for BH_4 deficiencies should be carried out in any newborns with plasma levels of 120μM phenylalanine or above, and in older children with neurological symptoms (Blau *et al*, 2001). The tests that are recommended for suspected BH_4 deficiencies are; 1) analysis of pterins in urine; 2) measurement of DHPR activity in blood from a Guthrie card (Guthrie and Susi, 1963); 3) BH_4 loading test; 4) analysis of pterins, folate and neurotransmitters plus metabolites in cerebrospinal fluid (CSF) (Hyland *et al*, 1993); and 5) enzyme activity measurements (Blau *et al*, 2001). The first two tests are essential for discerning which disorder a patient is likely to have. The BH_4 loading test allows differentiation between classical PKU and BH_4 variants, although false positives are possible in a subset of BH_4-responsive PAH deficient patients (Kure *et al*, 1999). Analysis of pterins, folate and neurotransmitters plus metabolites in CSF can then distinguish between mild and severe forms of the BH_4 deficiencies (Hyland *et al*, 1993), whilst individual enzyme assays will confirm these findings (Blau *et al*, 2001). Treatment is via BH_4, as well as L-dopa, carbidopa and 5-hydroxytryptophan, to treat neurotransmitter deficiencies in severe cases (Blau *et al*, 2001; Zurflüh *et al*, 2005).

1.11.1 *Autosomal Recessive GTP cyclohydrolase 1 deficiency*

Autosomal recessive GTP-CH deficiency is a rare and severe form of BH_4 deficiency, making up 21 of the 534 cases in the BIODEF database (http://www.bh4.org/BH4DatabasesBiodef.asp). Because GTP-CH1 is the first enzyme involved in synthesizing BH_4, virtually no pterins are synthesized in patients with this deficiency (Niederweiser *et al*, 1984a). Neopterin, biopterin, and 5-HT and DA metabolites 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) are all reduced in urine and CSF (Niederweiser *et al*, 1984b; Blau and Dhondt, 1998), although the relative proportions are in the normal range. Cultured fibroblasts from these patients when stimulated with cytokines are unable to respond with an

increase in BH_4 (Bonafe *et al*, 2001a). GTP-CH patients require high doses of BH_4 each day (1-2 doses of 5-20mg/kg per day), as well as full neurotransmitter precursor supplementation (Blau *et al*, 2001).

1.11.2 *6-Pyruvoyl tetrahydropterin synthase deficiency*

PTPS deficiency is the most common of all the BH_4 deficiencies, making up 56% (300 of 534 cases) of the reported cases in the BIODEF database (http://www.bh4.org/BH4DatabasesBiodef.asp). Mutations in PTPS mean that DHNTP cannot be converted to 6-pyruvoyl tetrahydropterin (figure 1.3), and thus DHNTP accumulates in the tissues of patients with this defect. DHNTP is easily dephosphorylated by pyrophosphatases, then oxidised to neopterin, and excreted (Blau *et al*, 2001). This oxidation product can be detected in urine and CSF using high performance liquid chromatography (Howells and Hyland, 1987), In PTPS deficiency neopterin levels are very high, and the neopterin to biopterin ratio is the greatest of the BH_4 deficiencies (Blau *et al*, 2001). Treatment for this enzymopathy is similar to GTP-CH deficiency (Blau *et al*, 2001; Wang *et al*, 2006).

1.11.3 *Sepiapterin reductase deficiency*

SR deficiency is the most recently described of the BH_4 metabolism disorders (Bonafe *et al*, 2001b), and so far only 17 cases have been reported (http://www.bh4.org/BH4DatabasesBiodef.asp). Patients present with psychomotor retardation, dystonia, and also extremely low concentrations of DA and 5-HT metabolites, but no HPA occurs with SR deficiency, and normal pterin levels are found in urine (Bonafe *et al*, 2001b). However, high levels of biopterin and dihydrobiopterin are reported in CSF, and investigation of cultured skin fibroblasts reveals a lack of SR activity (Bonafe *et al*, 2001b). Also, an accumulation of sepiapterin is noted in CSF (Zorzi *et al*, 2002a). It is proposed that because of the lack of SR in this disorder, 6PTP generated by PTPS can be reduced by AR or CR to dihydrobiopterin, and then reduced further to BH_4 by DHFR. However, DHFR expression is high in the liver and low in the brain (Kaufman, 1991), so therefore the BH_4 deficiency may only occur in the brain, and is why normal phenylalanine metabolism is observed (Bonafe *et al*, 2001b). Treatment for SR deficiency is via BH_4 and neurotransmitter precursors L-Dopa (plus carbidopa) and 5-hydroxytryptophan (Zorzi *et al*, 2002a; Neville *et al*, 2005).

1.11.4 *Dihydropteridine reductase deficiency*

As mentioned earlier in section 1.11, a DHPR deficiency was the first reported case of an HPA resulting from BH_4 deficiency (Kaufman *et al*, 1975). A total of 164 DHPR patients currently make up 31% of the 534 BH_4 deficiencies listed in the BIODEF database, making it the second most prevalent of these disorders (http://www.bh4.org/BH4DatabasesBiodef.asp). Quinonoid dihydrobiopterin is an extremely unstable intermediate and rapidly oxidises to the more stable dihydrobiopterin under physiological conditions (Davis *et al*, 1988). Therefore, in the absence of DHPR a build up of dihydrobiopterin is observed, and patients excrete high levels of this pterin, typically 80% above the normal range (Dhondt *et al*, 1981).

However, BH_4 levels in urine and CSF appear to be within the normal range in these individuals (Hyland and Heales, 1993; Ponzone *et al*, 2004). This is in contrast to the neurotransmitter profiles, which show DA and 5-HT to be impaired (Butler *et al*, 1978). A possible reason for this could be that dihydrobiopterin, which is also elevated in CSF (Ponzone *et al*, 2004) may inhibit the aromatic amino acid mono-oxygenases (Nagatsu *et al*, 1972, Heales and Hyland, 1990), which could lead to decreased concentrations of DA and 5-HT, as well as the observed HPA.

The clinical course of DHPR deficiency resembles GTP-CH and PTPS, in terms of symptoms as well as severity. Patients require L-Dopa, carbidopa and 5-hydroxytryptophan, although BH_4 supplementation is not necessary (Blau *et al*, 2001). Furthermore, they require folinic acid therapy (Irons *et al*, 1987; Woody *et al*, 1989) due to a build up of q-BH_2. Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20), an enzyme involved in folate biosynthesis, can convert q-BH_2 to BH_4 (Kaufman, 1991). However, in DHPR deficiency excess q-BH_2 then competes for MTHFR, such that folate biosynthesis is impaired (Kaufman, 1991). Folinic acid supplementation can reverse some of the effects of impaired folate metabolism in DHPR deficiency (Irons *et al*, 1987; Woody *et al*, 1989).

1.11.5 *Dopa responsive dystonia*

Dopa responsive dystonia (DRD), also known as Segawa's disease or Hereditary Progressive dystonia, is an autosomal dominant GTP-CH deficiency. It was first

the disorder wasn't properly elucidated until 1994 (Ichinose *et al*, 1994). It is most often caused by a heterozygous mutation of the *GCH* gene that leads to a partial BH_4 deficiency, but is not accompanied by HPA (Ichinose *et al*, 1994). Usually the symptoms of this disorder are not as severe as the recessively inherited form, with patients presenting at around 6 years of age, starting with a postural dystonia that fluctuates diurnally. This progresses to all limbs in the following years, and is accompanied by tremor and rigidity amongst other clinical signs (Segawa *et al*, 2003). Mental retardation is not seen in DRD, unlike in the autosomal recessive form of GTP-CH deficiency, and patients respond well to L-Dopa treatment, usually without side effects (Segawa *et al*, 2003).

So far there are 98 cases of DRD in the International database of mutations causing tetrahydrobiopterin deficiencies (BIOMDB)(Blau and Thöny, 1998). Most of the investigations into DRD have so far suggested that the reason why autosomal dominant mutations give rise to BH_4 deficiency is because the mutant has a dominant negative effect on the wild type enzyme. Hwu *et al* show that when mutant DRD GTP-CH and wild type enzymes are expressed in eukaryotes, the presence of the mutant decreases the wild type protein and activity levels (Hwu *et al*, 2000). The net result of this is a partial deficiency in BH_4. TH activity and levels of the DA metabolite HVA are decreased in DRD, however TRH activity appears normal, and reports of 5-HT concentrations in DRD are inconsistent (Nygaard 1993; Takahashi *et al*, 1994). TH activity is thought to be attenuated because under normal conditions intracellular concentrations of BH_4 are close to the K_m of the enzyme (approximately 500μM)(Levine *et al*, 1981). TH could therefore be sensitive to changes in BH_4 that occur in DRD, leading to decreased enzyme activity (Blau *et al*, 2001). In addition, microdialysis studies show that BH_4 can stimulate DA release (Koshimura *et al*, 1990), suggesting that in conditions where BH_4 is limiting such as DRD, normal release of DA into the synapse may be prevented.

Although HPA is not characteristically observed in DRD under normal conditions, stressing of the phenylalanine hydroxylating system with an oral dose of phenyalanine does cause HPA (Hyland *et al*, 1997). Phenylalanine levels are increased for prolonged periods, and can be normalised with BH_4 pre-treatment. This

is a useful non-invasive method of diagnosing DRD, and is preferable to measuring CSF pterin profiles via lumbar puncture (Hyland *et al*, 1997).

1.11.6 *Impaired nitric oxide metabolism in tetrahydrobiopterin deficiencies*

Analysis of nitrite and nitrate levels as an index of NO production (Clelland *et al*, 1996) show that in patients with inborn errors of BH_4 metabolism, NO generation may be impaired (Heales *et al*, 1999a; Zorzi *et al*, 2002b). Furthermore, in an animal model of partial BH_4 deficiency, the *hph-1* mouse (see section 1.12), NO metabolism is attenuated (Brand *et al*, 1996; Canevari *et al*, 1999), suggesting that even a partial deficiency can result in impaired NO production. These observations appear to contrast with the finding that NOS has a low K_m for BH_4 of typically 0.02μM (Giovanelli *et al*, 1991), which implies that BH_4 should not limit NO production from NOS. However, it has been demonstrated that some BH_4 metabolites, including dihydrobiopterin can competitively inhibit NOS (Jones *et al*, 2001), which are present in high quantities in CSF in some of the BH_4 deficiencies, such as DHPR deficiency (Hyland and Heales, 1993) and PTPS deficiency (Blau *et al*, 2001). BH_4 deficiencies may also impact on the availability, and the affinity of the substrate arginine for NOS. In the absence of exogenous BH_4, the K_m of nNOS for arginine is elevated in the *hph-1* mouse (Brand *et al*, 1995). In addition, arginine uptake is dependent on the amount of BH_4 present in the cell (Schwartz *et al*, 2001). These findings suggest that arginine may become limiting for the NOS reaction in BH_4 deficient states. Furthermore, under low BH_4 concentrations the NOS reaction can become uncoupled (Cosentino *et al*, 1998), and BH_4 is important for dimerisation of NOS (Crane *et al*, 1998). Under these conditions the highly reactive peroxynitrite ($ONOO^-$) is formed from superoxide ($\cdot O_2^-$) and $NO\cdot$, which may contribute to the neurological damage seen in some of the BH_4 deficiencies (Tiefenbacher, 2001).

Although some patients respond well to BH_4 supplementation and monoamine neurotransmitter replacement therapy, there are a number that show severe mental retardation despite these treatment regimes and a low phenylalanine diet (Endres, 1985; Dudesek *et al*, 2001). Given the widespread distribution and role of NO in the brain (section 1.10), the consequences of a deficiency in BH_4 may have extensive implications.

1.11.7 *Acquired tetrahydrobiopterin disorders*

There are a number of conditions, both of the periphery and CNS, which are associated with changes in BH_4 metabolism. Due to its role in maintaining vascular tone via eNOS, BH_4 deficiencies are implicated with a number of diseases that have a pathogenesis in the cardiovascular system (reviewed in Alp and Channon, 2003; Channon, 2004). Disease states including diabetes, hypertension, and atherosclerosis have all been linked to an associated BH_4 deficiency, which may give rise to endothelial dysfunction via eNOS uncoupling, and oxidative stress (Heitzer *et al*, 2001; Bonetti *et al*, 2003; Boulden *et al*, 2006).

In the CNS, BH_4 deficiency may contribute to the etiology of some disorders, however an excess of BH_4 is implicated in others. Decreased levels of BH_4 or metabolites have been observed in Parkinson's disease (PD)(Lovenberg *et al*, 1979; Williams *et al*, 1980), Alzheimer's disease (AD)(Barford *et al*, 1984; Casal *et al*, 2003), Huntington's disease (Williams *et al*, 1980), schizophrenia (Richardson *et al*, 2005), bipolar affective disorder (Hoekstra *et al*, 2006) and depression (Bottiglieri *et al*, 1992; Newman and Holden, 1993). Furthermore, in AD, PD and multiple sclerosis (MS) there is evidence that increased oxidative stress may be involved (Heales *et al*, 1999b). Increased NO generation has been implicated in these disorders via nitrite and nitrate measurement (Johnson *et al*, 1995; Qureshi *et al*, 1995; Tohgi *et al*, 1998). Decreased expression of guanylate cyclase, which mediates some of the effects of NO, is also reported in MS, AD and Creutzfeldt-Jacob post-mortem brains (Baltrons *et al*, 2004).

Other literature points to the possible cytotoxic effects of BH_4. It is reported that BH_4 may selectively mediate dopaminergic cell death in animal models of PD (Kim *et al*, 2003; Choi *et al*, 2003, 2004), possibly by causing mitochondrial dysfunction (Choi *et al*, 2006; Lee *et al*, 2006). In addition, blockade of BH_4 synthesis may be protective in animal models of ischaemic brain injury (Cho *et al*, 1999; Kidd *et al*, 2005). However, experiments in rat nigral slice cultures show that sepiapterin, a precursor of BH_4, can protect against MPP+ mediated cell death (Madsen *et al*, 2003). Therefore, the role of BH_4 in neurodegeneration needs further evaluation.

1.12 The *hph-1* mouse

The *hph-1* mouse is a model system for the study of BH_4 deficient states (Hyland *et al*, 2003). It is partially deficient in BH_4 due to a mutation that affects GTP-CH expression (Gütlich *et al*, 1994). It was originally developed via ethylnitrosourea mutagenesis as a model for HPA (Bode *et al*, 1988), however it was discovered that levels of phenylalanine normalise after 3 weeks of age (McDonald *et al*, 1988). The history and the biochemistry of the *hph-1* mouse will be discussed in greater depth in chapter 3.

1.13 Gene therapy

Gene therapy is the insertion of genetic material, most often using a viral vector, into an organism for the treatment of disease. It was initially conceived as a remedy for single-gene inherited defects (Kay *et al*, 1994) such as Duchenne muscular dystrophy (Thioudellet *et al*, 2002), however the scope has now broadened to treatment for a number of other disorders including cancer (Lowenstein, 1997), cardiovascular disease (Isner, 2002), infectious disease (Bunnell and Morgan, 1998) and neurodegenerative disease (Baekelandt *et al*, 2000). Viral vectors are currently the most commonly used way of introducing therapeutically beneficial genetic material into the patient, however other strategies are being developed. The background to gene therapy, along with the types of gene therapy and the vectors used are discussed in greater depth in chapter 5.

1.14. AIMS

The aims of this thesis are:

- To further characterise the biochemistry of the *hph-1* mouse, in both the brain and cultured cortical astrocytes.
- To use gene transfer to correct BH_4 and NO metabolism in *hph-1* astrocytes.
- To evaluate the mechanisms behind any improvement in BH_4 or NO metabolism in *hph-1* astrocytes following gene transfer.
- To carry out preliminary experiments for future *in vivo* gene transfer work in the *hph-1* mouse.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd., Poole, UK unless stated otherwise.

2.1. High Performance Liquid Chromatography (HPLC)

2.1.1 Quantification of tetrahydrobiopterin by reverse-phase HPLC coupled with electrochemical detection

The general principle of reverse-phase High-Performance (or High-pressure) Liquid Chromatography (HPLC) is the separation and elution of a sample between an aqueous mobile phase and organic solid phase (HPLC column). Once the sample has passed through the HPLC column and been separated, coulometric electrochemical detection (ECD) can be used to quantify the desired chemical species, such as BH_4, which is done by oxidation and reduction of the sample at a dual electrode analytical cell. Electroactive compounds such as BH_4 are oxidised at electrode 1, and then the oxidation products of BH_4 and compounds with similar properties are reduced at electrode 2. The potential generated to reduce BH_4 is monitored using a detector, which can then be quantified by comparing the signal intensity with a standard of known concentration. The potentials applied at electrodes 1 and 2 can be varied so that the applied voltages and resulting background currents give rise to the optimal signal: noise ratio, so that the detection of BH_4 with lowest background noise is optimal. To determine the optimal voltages at electrodes 1 and 2, voltammograms were performed which are shown in figure 2.1. To do this electrode 2 was maintained at – 0.05 μamps, whilst 50nM BH_4 standards were run and the potential at electrode 1 changed by increments of 50mV until the optimal standard peak height, with lowest background noise was obtained. The optimal potential at electrode 1 was determined to be + 0.5 μamps, which then served as a constant value to perform a voltammogram at electrode 2 (reduction electrode). Electrode 2 potentials were decreased by 50mV until maximal BH_4 levels were detected at –0.05 μamps.

The oxidation and reduction of electroactive species, such as BH_4, at electrodes 1 and 2 allows for selective analysis of these compounds. Not all compounds can be oxidised by electrode 1, which means that these pass through electrode 2 undetected.

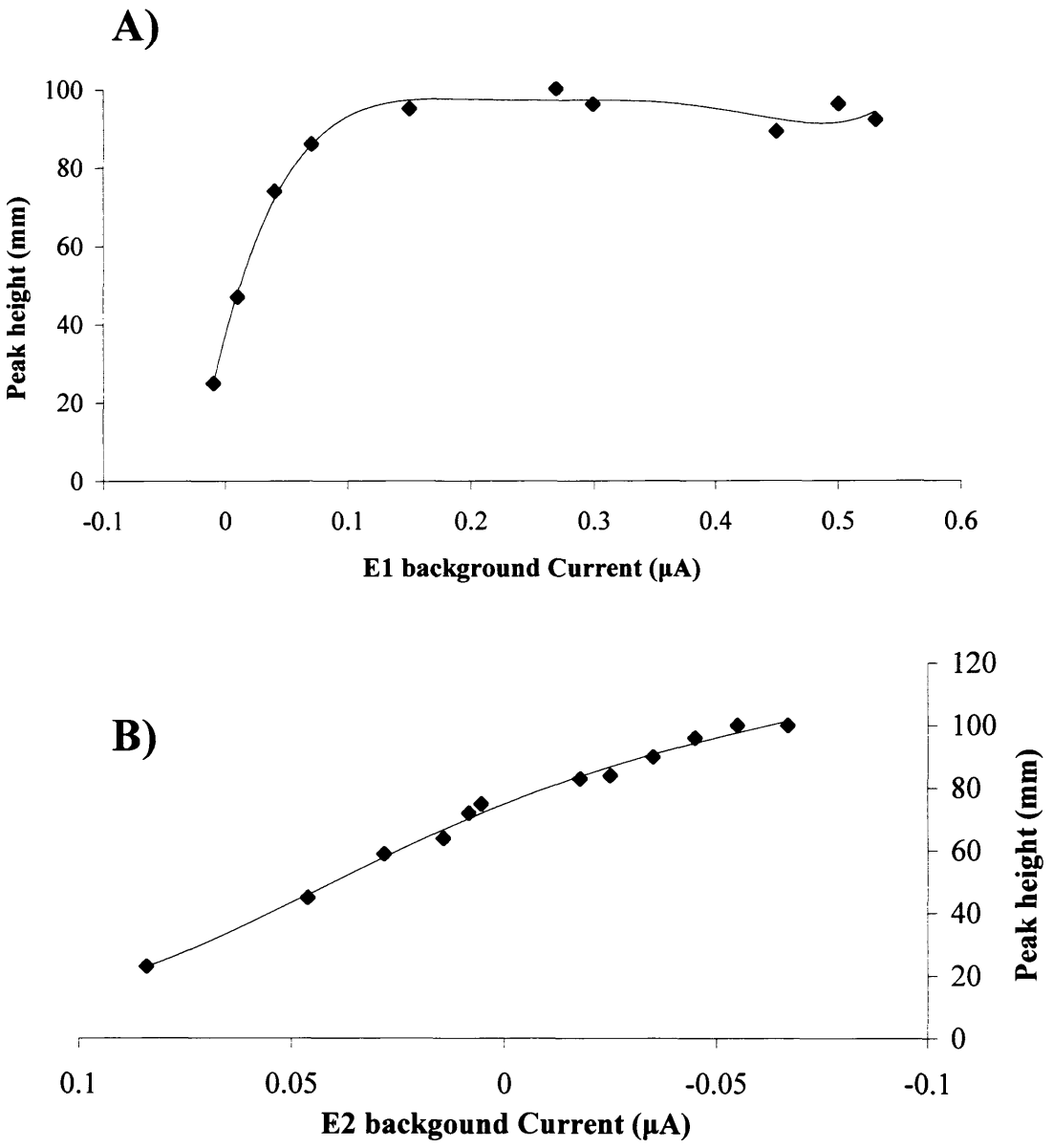


Figure 2.1. BH_4 voltammograms.

50pmol/ml BH_4 standards were measured using reverse phase HPLC coupled with ECD.

A) Determination of the optimum potential and resulting background current of the oxidising electrode 1 (E1). The reduction electrode 2 (E2) was maintained at a constant potential of –0.05 µamps (µA) whilst potentials at electrode 1 were varied until the optimal peak height, with lowest background noise was obtained. This was determined to be +0.5 µA for electrode 1. **B)** The process was repeated for electrode 2, with electrode 1 kept at a constant of +0.5 µA. The optimum potential at electrode 2 was –0.05 µA.

In addition, once oxidised not all compounds can be reduced back by electrode 2, which provides further specificity. This specificity coupled with the renowned sensitivity of ECD allows for detection limits in this assay of around 1pmol of BH_4.

2.1.2 Method of separation of tetrahydrobiopterin

BH_4 was measured based on the method of Howells and Hyland (1987) by reverse phase HPLC coupled with ECD. Figure 2.2 shows a schematic representation of the chromatographic system, which is composed of a Jasco PU-1580 pump (Jasco, Great Dunmow, Essex, UK), a Rheodyne Inc. (Rohnert Park, Ca, USA) model 7010 injection valve with a 50µl injection loop, guard column GTS-E03501 (HPLC technology, Welwyn Garden City, Herts, UK), an HPLC technology Technisphere octadecasilyl reverse phase column with particle size 5µm, 4.6mm x 260mm (HPLC technology), heated to 37°C by a Jetstream 2 plus column heater (Jasco). BH_4 was detected by an ESA 5100A detector (ESA Analytical, Aylesbury, UK) using an ESA 5011 dual electrode analytical cell. Mobile phase consisted of 50mM sodium acetate, 5mM citric acid (both from BDH Laboratory supplies Ltd., Poole, UK), 48µM ethylenediaminetetraacetic acid (EDTA) and 160µM dithioerythritol (DTE), prepared in 18.2 MΩ Millipore Q H_2O. All reagents used were HPLC grade quality. Mobile phase was run to waste through the HPLC system overnight at 0.3ml/minute to equilibrate and allow the electrochemical detector to stabilise, before being run at 1.3ml/minute for BH_4 measurements. Stock 500µM tetrahydrobiopterin hydrochloride standards (Schircks Laboratories, Jona, Switzerland) were made up in ice cold 18.2 MΩ Millipore Q H_2O containing 6.5mM DTE and 2.5mM diethylenetriaminepentaacetic acid (DETAPAC), and aliquots stored at -70°C. Aliquots were diluted to 50nM in 6.5mM DTE and 0.25mM DETAPAC to make a working standard. Peaks were recorded using a ThermoFinnigan Chromjet integrator (Anachem, Luton, UK) and peak heights determined by hand. To validate detection of BH_4 using this method, a standard curve was produced showing linearity to 50nM of a synthetic BH_4 standard (R^2 0.9967)(figure 2.3). A schematic chromatogram showing a BH_4 peak is shown in figure 2.4.

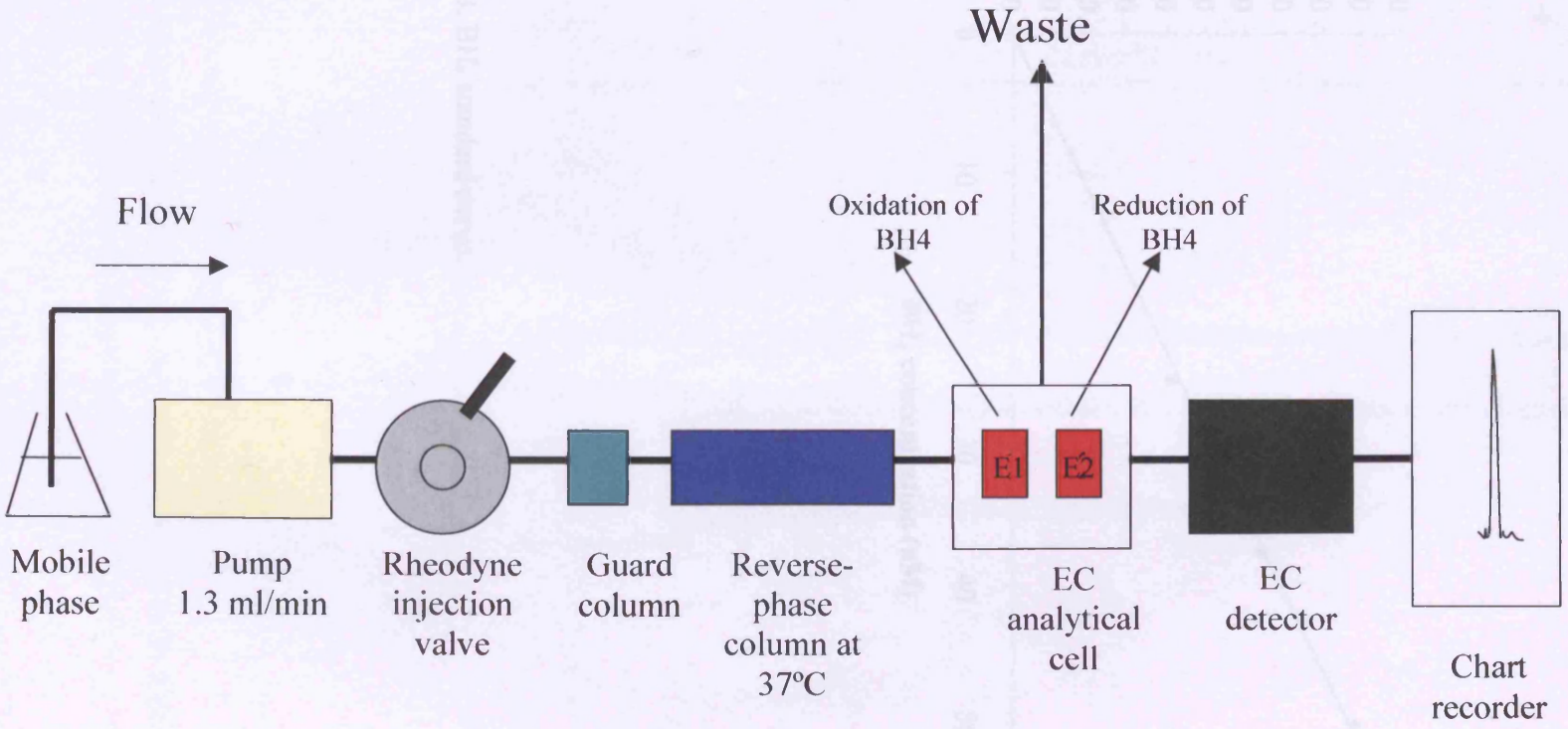


Figure 2.2. Schematic representation of reverse-phase HPLC system used to measure BH_4.

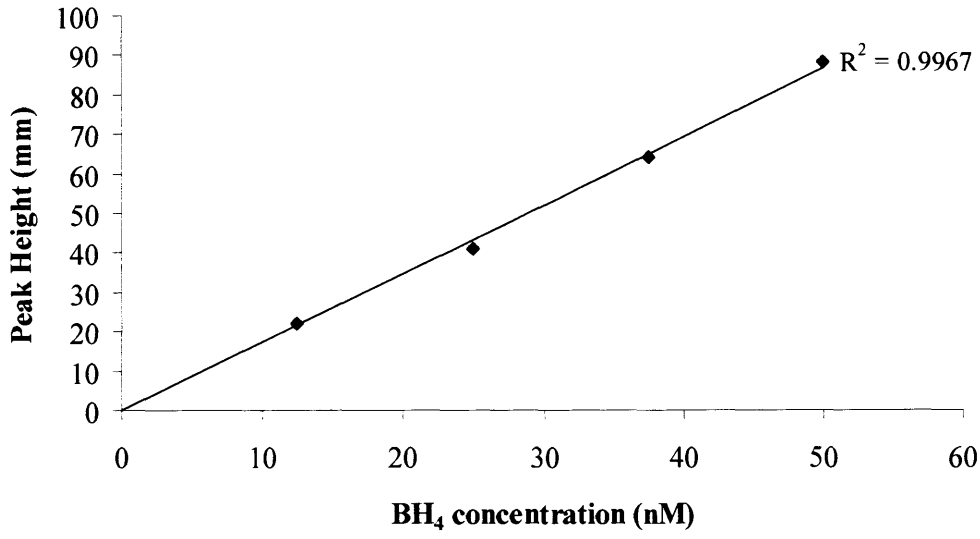


Figure 2.3. BH_4 standard curve.

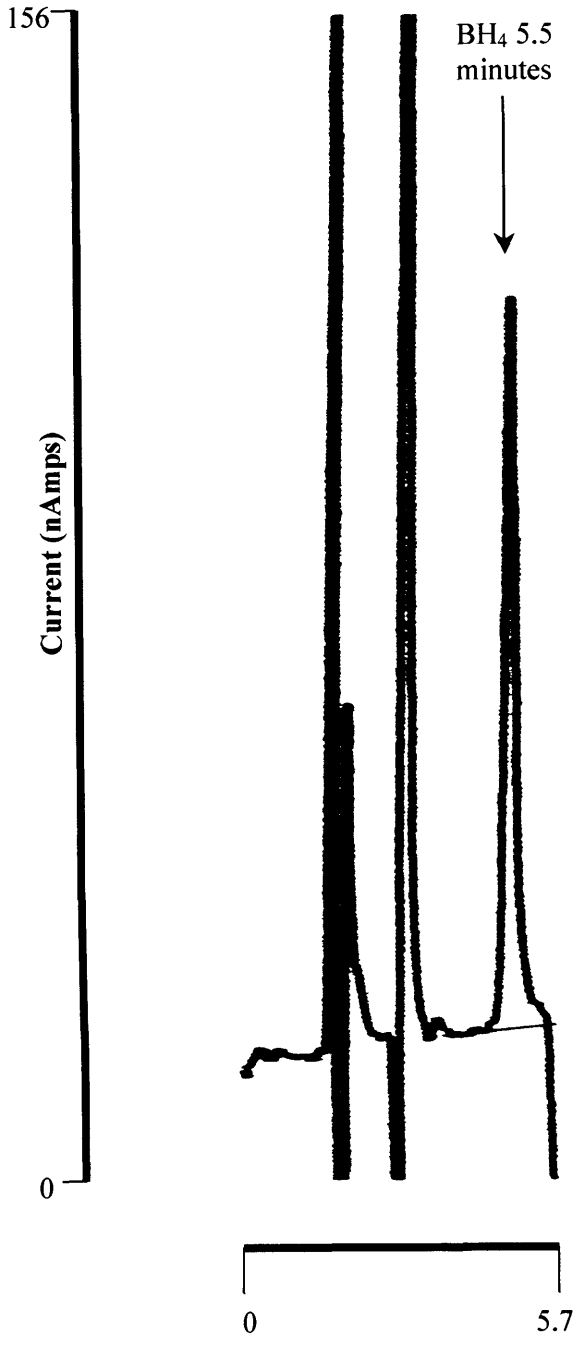


Figure 2.4. Chromatogram of a 50nM BH_4 standard.

BH_4 was measured using reverse phase HPLC coupled with ECD. BH4 has a retention time of 5.5 minutes at a temperature of 35°C.

2.1.3 Quantification of monoamines by reverse-phase HPLC coupled with electrochemical detection

The monoamines noradrenaline (NA), dopamine (DA), 3,4-dihydroxy-phenylacetic acid (Dopac), homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5-HIAA) were measured using reverse-phase HPLC coupled with ECD. The general principles of reverse-phase HPLC with ECD used to detect BH_4, also apply for detection of monoamines. However, instead of the oxidation and reduction of BH_4 that occurred at electrodes 1 and 2, in this method both electrodes function in an oxidising capacity. Electrode 1 was set at + 50mV to oxidise and eliminate molecules with lower oxidation potentials than the monoamines being quantified. Voltammograms were carried out for electrode 2 to determine the optimal oxidising potential and resulting background current for each of the monoamines. Individual 500nM standards of NA, DA, Dopac, HVA, MHPG and 5HIAA were run with background currents set between + 100mV and + 500mV. Peak heights were measured and plotted to see which voltage gave the optimal peak height, with the lowest background noise. Detection of the monoamines was optimal at a voltage of + 400mV (figure 2.5). A 500nM standard containing NA, DA, Dopac, HVA, MHPG and 5HIAA was also measured at varying electrode 2 voltages between + 100mV and + 500mV. Peak heights of all the standards were measured and combined to give total peak height, then plotted against voltage. This also gave an optimal peak height of + 400mV (figure 2.6). In order to establish which of the peaks in the mixed standard corresponded to each monoamine, individual 500nM and 1000nM standards were run of each neurotransmitter or metabolite, and were compared to the retention time of the mixed standard to discern elution time.

2.1.4 Method of separation of the monoamines

The monoamines NA, DA, Dopac, HVA, MHPG and 5HIAA were measured based on the method of Niederwieser *et al* (1984a). Figure 2.7 shows a schematic representation of the chromatographic system, which is comprised of a Jasco PU-1580 pump (Jasco, Great Dunmow, Essex, UK), a Rheodyne Inc. model 7010 injection valve with a 50µl injection loop (Rohnert Park, Ca, USA), an HPLC technology Technisphere octadecasilyl reverse phase column with particle size 5µm, 4.6mm x 260mm (HPLC technology, Welwyn Garden City, Herts, UK), heated to 35°C by a Jetstream 2 plus column heater (Jasco, Great Dunmow, Essex, UK). An

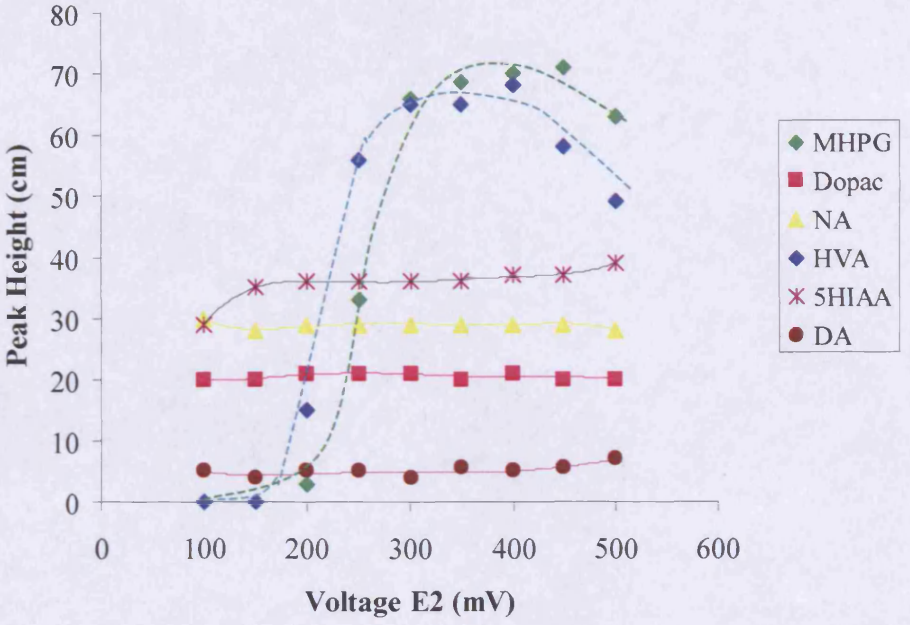


Figure 2.5. Individual monoamine voltammograms at electrode 2.

500pmol/ml standards of dopamine, homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA), noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (Dopac) and 3-methoxy-4-hydroxyphenolglycol (MHPG) were measured using reverse phase HPLC coupled with coulometric detection.

Optimal peak height was determined using a voltammogram by changing the potential at electrode 2 (E2). Peak heights of all monoamines detected were measured, and then plotted against voltage at E2. The optimal voltage at E2 that produced the greatest total peak height with lowest background noise was + 400mV.

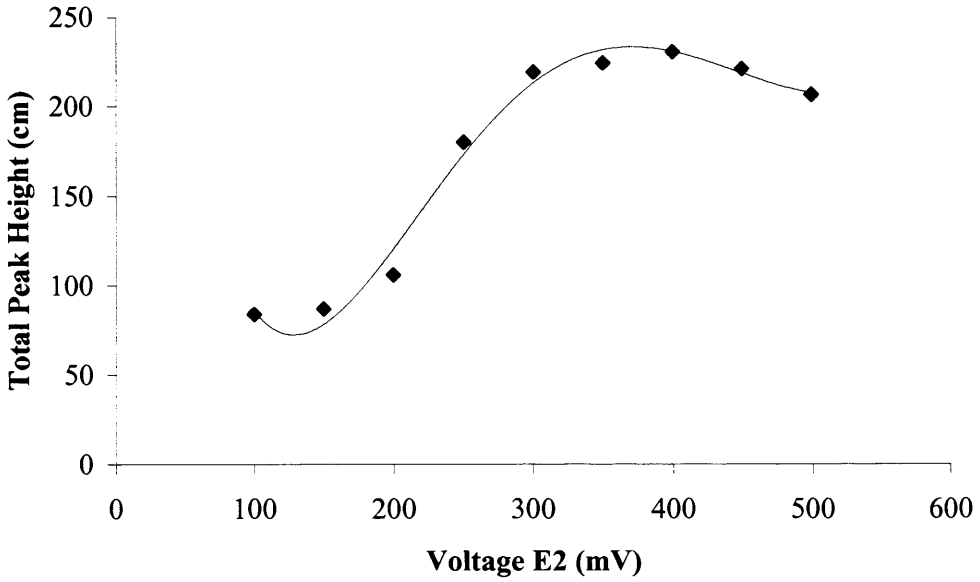


Figure 2.6. Combined monoamine voltammogram at electrode 2.

500pmol/ml monoamine standards were measured using reverse phase HPLC coupled with coulometric detection.

Optimal peak height was determined using a voltammogram by changing the potential at electrode 2 (E2). Peak heights of all monoamines detected were measured and added to give a sum of the total peak heights, and plotted against voltage at E2. The optimal voltage at E2 that produced the greatest total peak height with lowest background noise was + 400mV.

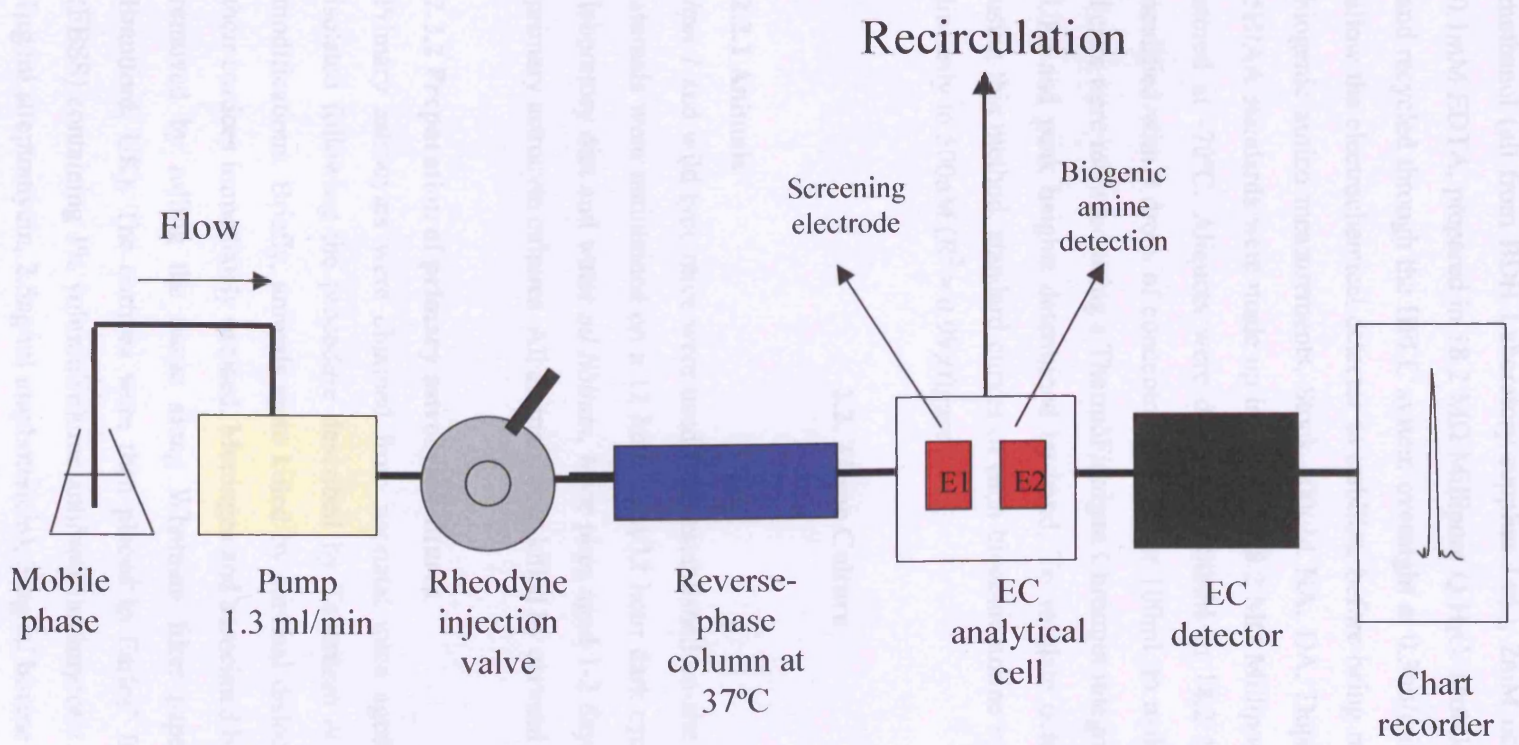


Figure 2.7. Schematic representation of reverse-phase HPLC system used to measure biogenic amines.

electrochemical ESA 5100A detector (ESA Analytical) in combination with an ESA 5010 dual electrode analytical cell was used to detect the monoamines. Mobile phase consisted of 20mM sodium acetate, 12.5mM citric acid, 16% volume:volume methanol (all from BDH Laboratory supplies Ltd.), 2mM octanesulfonic acid, and 0.1mM EDTA, prepared in 18.2 MΩ Millipore Q H_2O. Mobile phase was circulated and recycled through the HPLC system overnight at 0.3ml/minute to equilibrate and allow the electrochemical detector to stabilise, before being run at 1.3ml/minute for biogenic amine measurements. Stock 500μM NA, DA, Dopac, HVA, MHPG and 5HIAA standards were made up in ice cold 18.2 MΩ Millipore Q H_2O and aliquots stored at -70°C. Aliquots were diluted to 500nM in 18.2 MΩ Millipore Q H_2O acidified with 3 drops of concentrated HCl per 100ml, to make a working standard. Peaks were recorded using a ThermoFinnigan Chromjet integrator (Anachem, Luton, UK) and peak heights determined by hand. To validate detection of monoamines using this method, standard curves of each biogenic amine were produced showing linearity to 500nM ($R^2 > 0.99$)(figure 2.8).

2.2. Tissue Culture

2.2.1 Animals

hph-1 and wild type mice were used from established on-site breeding colonies. All animals were maintained on a 12 hour light/12 hour dark cycle, and fed with stock laboratory diet and water *ad libitum*. Mice pups aged 1-2 days old were used for all primary astrocyte cultures. All animals were killed by cervical dislocation.

2.2.2 Preparation of primary astrocyte cultures

Primary astrocytes were obtained from neonatal mice aged up to 1-2 days, and isolated following the procedure described by Tabernero *et al.* (1993) with some modifications. Briefly, animals were killed by cervical dislocation, decapitated and their cortices immediately excised. Meninges and associated blood vessels were then removed by rolling the tissue along Whatman filter paper 1 (Whatman PLC, Brentford, UK). The cortices were then placed in Earles' Balanced Salt Solution (EBSS) containing 1% volume/volume antibiotic antimycotic (10units/ml penicillin, 1μg/ml streptomycin, 2.5ng/ml amphotericin), 3mg/ml bovine serum albumin (BSA)

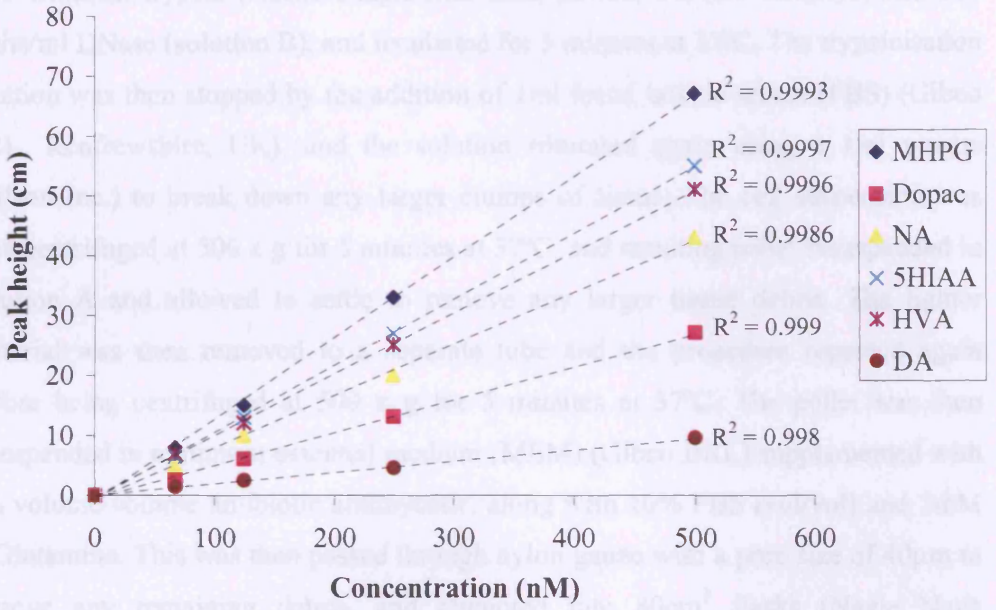


Figure 2.8. Standard curves of dopamine (DA), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA), noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (Dopac) and 3-methoxy-4-hydroxyphenolglycol (MHPG). Regression analysis calculates that all standard cures show linearity ($R^2 > 0.99$).

and 75Units/ml deoxyribonuclease 1 (DNase) (EC 3.1.21.3) (solution A). The cortices were roughly chopped using curved scissors and then gently triturated using a 1ml pipette (Gilson Inc., WI, USA) to further break up the tissue. This was then transferred to a tube and centrifuged at 500 x g for 5 minutes at 4°C. The pellet was retained and resuspended in a solution made of solution A, additionally containing 27.5 Units/ml trypsin (Roche Diagnostics Ltd., Lewes, UK)(EC 3.4.21.4) and 567 Units/ml DNase (solution B), and incubated for 5 minutes at 37°C. The trypsinisation reaction was then stopped by the addition of 1ml foetal bovine serum (FBS) (Gibco BRL, Renfrewshire, UK), and the solution triturated again using a 1ml pipette (Gilson Inc.) to break down any larger clumps of tissue. The cell suspension was then centrifuged at 500 x g for 5 minutes at 37°C, and resulting pellet resuspended in solution A and allowed to settle to remove any larger tissue debris. The lighter material was then removed to a separate tube and the procedure repeated again before being centrifuged at 500 x g for 5 minutes at 37°C. The pellet was then resuspended in minimum essential medium (MEM) (Gibco BRL) supplemented with 1% volume/volume antibiotic antimycotic, along with 10% FBS (vol/vol) and 2mM L-Glutamine. This was then passed through nylon gauze with a pore size of 40μm to remove any remaining debris, and aliquoted into 80cm^2 flasks (Nagle Nunc International, Naperville, Il, USA). Typically 2-3 brains were allocated per flask and in a volume of 10ml/flask. Cells were placed in an incubator at 37°C, with 5% CO_2, 95% air and 95% humidity. The medium was changed the next day, and then twice a week thereafter.

2.2.3. Shaking and plating of cells

Once cells had reached 90-100% confluence in the flasks (usually around day 8-10), they were shaken in an attempt to remove microglia or fibroblast cells left from the culture. They were placed in a Heidolph 1010 shaker (LabPlant, Huddersfield England) within an incubator (for conditions see above) and shaken at 200 rpm overnight. The medium was removed and then replaced with fresh medium.

After 13 days in culture the cells were ready for treatment. Each flask of astrocytes was trypsinised with 10 ml trypsin/EDTA solution (0.05% weight:volume trypsin and 0.02% weight:volume EDTA) for 5 minutes at 37°C. The trypsinisation reaction was stopped by the addition of 1ml cold FBS per flask. Cells were collected and

flasks washed with a further 10ml of warm Hanks' Balanced Salt Solution (HBSS), before being centrifuged at 2000 x g at 4°C for 5 minutes. The resulting pellet was resuspended in MEM supplemented with 1% volume/volume antibiotic antimycotic, along with 10% volume/volume FBS and 2mM L-Glutamine, and then cells counted using a haemocytometer. They were seeded at a density of 1 x 10^6 cells per well on poly-D-ornithine coated 6 well plates (Nagle Nunc International) for BH_4, and NO_2^- and NO_3^- measurements, or 5 x 10^5 cells per well on poly-D-ornithine coated 12 well plates (Nagle Nunc International) for Western blotting. During counting astrocytes were tested for exclusion of trypan blue to show that cell viability was higher than 90%. Cells were given 24 hours to settle before any treatment took place.

2.2.4. Treating cells

After 24 hours settling on 6 well plates, the medium was removed from each well and replaced with phenol red-free MEM supplemented with 2mM L-Glutamine, along with any individual treatments. The cells were then replaced back into the incubator and left for defined periods of time depending on the treatment. The methods section 4.3.5 describes the various treatment periods for astrocytes.

2.3. Sample Preparation

2.3.1. Preparation of brain cytosol fraction

Wild type and *hph-1* brain tissue was homogenised to 35% weight:volume in 0.1M Tris HCl pH 7.6 buffer, using a 2ml Kontes tissue grinding pestle and mortar (Kontes Glass Company, NJ, USA). The homogenate was centrifuged at 48,000rpm (100,000 x g) for 1 hour at 4°C. The supernatant cytosolic fraction was snap frozen in a sealed eppendorf tube and stored at -80°C until it was used in experiments.

2.3.2. Preparation of tissue for tetrahydrobiopterin and monoamine measurement

For BH_4 measurement wild type and *hph-1* brain tissue was homogenised to 25% weight:volume in ice cold 0.1M perchloric acid (PCA) (BDH Laboratory supplies Ltd.) buffer, containing 6.5mM DTE and 2.5mM DETAPAC, using a 2ml Kontes tissue grinding pestle and mortar (Kontes Glass Company). Homogenates were centrifuged at 15,000 x g for 5 minutes, and the supernatant was used for measuring

BH_4 content, by reverse-phase HPLC as described in section 2.1. Results were expressed as pmol/mg protein.

For monoamine neurotransmitter measurements, brain tissue was treated in the same way as for BH_4 measurement, but homogenisation buffer also contained 0.1mM EDTA. Results were expressed as pmol/mg protein.

2.3.3. Harvesting and preparation of astrocytes for tetrahydrobiopterin measurement

Following treatment, cells were harvested by one of two different methods for tetrahydrobiopterin measurement. In initial experiments cells were trypsinised in trypsin/EDTA solution (0.05% weight:volume trypsin and 0.02% weight:volume EDTA) for 5 minutes at 37°C, and the reaction stopped using 75µl FBS. The plate was washed using 750µl HBSS and added to collected cells, and then centrifuged at 2000 x g for 5 minutes. The supernatant was discarded and the pellet resuspended in 50µl of HBSS, from which 10µl was taken for protein analysis (section 2.9.), and the rest diluted 1:2 by PCA buffer (0.1M PCA, 6.5mM DTE and 2.5mM DETAPAC). This was triturated using a 1ml pipette (Gilson Inc.) and then centrifuged at 15,000 x g for 5 minutes, and the supernatant injected onto the HPLC system for detection. However, due to the instability of BH_4 (Howells *et al*, 1986) it was proposed that the trypsinisation procedure could degrade much of the BH_4 present in the cells. Therefore an alternative harvesting method was investigated, which involved putting the PCA buffer directly onto the cells after the medium had been aspirated. Cells were treated with 150µl of PCA buffer then scraped to remove the monolayer using a cell scraper (Corning, New York, USA). Each well was washed with 150µl HBSS and added to the collected cells, then centrifuged at 15,000 x g for 5 minutes. The supernatant was used for HPLC analysis (section 2.1), and the pellet for protein concentration (section 2.9.). Figure 2.9 shows the effect of these scraping techniques on overall BH_4 concentration, in both stimulated and control wild type astrocytes. It was shown using a Students t-test that direct PCA scraping significantly increases BH_4 yield when compared to trypsinised cells, by 5 times in control ($p = 0.001$, $n = 4$), and by 7 times in treated wild type astrocytes ($p = 0.009$, $n = 4$). Therefore this method was used for all BH_4 measurements in astrocytes. Results were expressed as pmol/mg protein.

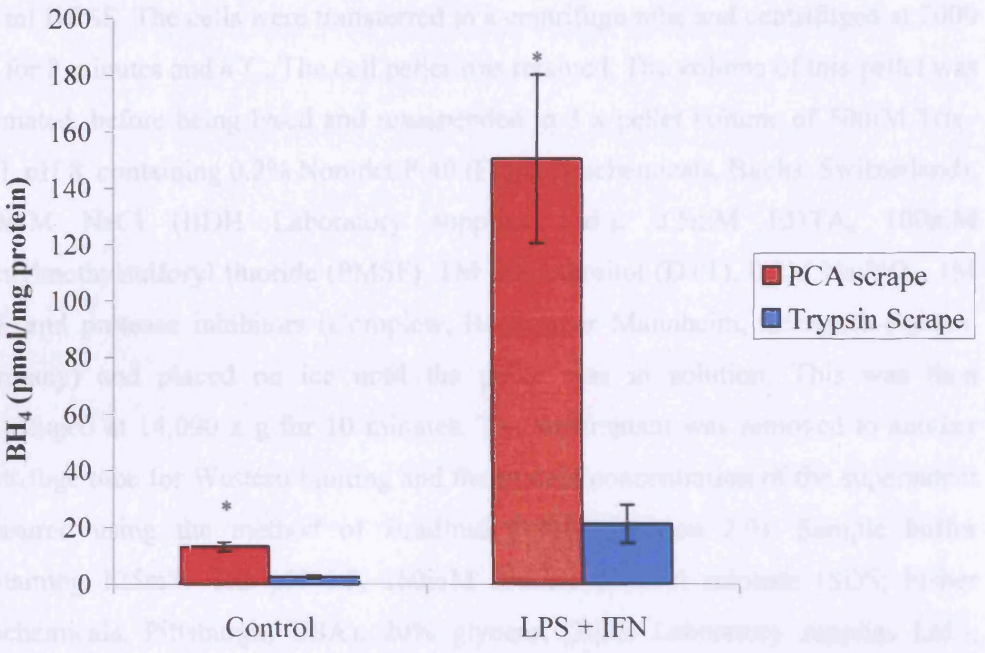


Figure 2.9. Comparison of different scraping methods on BH_4 levels in control and LPS + IFN-γ (100 U/ml LPS + 1μg/ml IFN) stimulated wild-type astrocytes (see section 2.3.4.). Cells were harvested either by trypsinisation (trypsin scrape), or by adding perchloric acid buffer (PCA) straight onto the cells (PCA scrape).
BH_4 was measured by reverse-phase HPLC coupled with ECD. Data is expressed as mean ± SEM of independent cell culture preparations (n = 4). * = $p < 0.01$ vs. trypsin scrape, as judged using Students t-test.

2.3.4. Harvesting and preparation of astrocytes for Western blotting

Astrocytes were grown as described in (section 2.2) and seeded at a density of 5 x 10^5 in 0.5 ml media on 12 well plates (Corning). Cells were harvested in 0.5ml ice cold HBSS using a cell scraper (Corning), and each well was washed with a further 0.5 ml HBSS. The cells were transferred to a centrifuge tube and centrifuged at 2000 x g for 3 minutes and 4°C. The cell pellet was retained. The volume of this pellet was estimated, before being lysed and resuspended in 3 x pellet volume of 50mM Tris–HCl, pH 8, containing 0.2% Nonidet P-40 (Fluka Biochemicals, Buchs, Switzerland), 180mM NaCl (BDH Laboratory supplies Ltd.), 0.5mM EDTA, 100mM phenylmethylsulforyl fluoride (PMSF), 1M dithiothreitol (DTT), 0.2M Na_3VO_4, 1M NaF and protease inhibitors (Complete, Boehringer Mannheim, Grenzach-Eyhlen, Germany) and placed on ice until the pellet was in solution. This was then centrifuged at 14,000 x g for 10 minutes. The supernatant was removed to another centrifuge tube for Western blotting and the protein concentration of the supernatent measured using the method of Bradford (1976) (section 2.9). Sample buffer containing 125mM Tris pH 6.8, 150mM sodium dodecyl sulphate (SDS; Fisher Biochemicals, Pittsburgh, USA), 20% glycerol (BDH Laboratory supplies Ltd.), 15μM bromophenol blue, and 4% β-mercaptoethanol, was added to double the volume of the supernatant. This was then centrifuged at 14,000 x g for 10 minutes and stored at -20°C until Western blotting was carried out.

2.4. Measurement of Tetrahydrobiopterin biosynthetic capacity

BH_4 biosynthetic capacity was measured following the method of Barford *et al.* (1984). Brain cytosolic fractions (section 2.3.1.) were incubated with $MgCl_2$ (final concentration 6mM), 0.1M Tris/0.04M KCl (pH 8.0), along with 6mM guanosine 5'-triphosphate (GTP) and 3mM NADPH to stimulate and support synthesis of BH_4. Blank (or basal) samples were incubated in the absence of GTP and NADPH. All samples (final volume 500μl) were then incubated for 3 hours at 37°C, at which time reactions were stopped by the addition of 0.1M PCA (BDH Laboratory supplies Ltd.) solution containing 6.5mM DTE and 2.5mM DETAPAC. Samples were then centrifuged at 15,000 x g for 5 minutes at 4°C, and the supernatant analysed for BH_4 content using HPLC coupled with ECD. Total BH_4 biosynthetic capacity was then determined as stimulated BH_4 minus basal BH_4, then expressed as pmol/hour, and finally related back to the amount of protein in the original homogenate or cytosolic

fraction and expressed as pmol/hour/mg protein. To validate the use of this method a standard curve was produced showing linearity of biosynthetic capacity with increasing amounts of cytosol (figure 2.10).

2.5. Measurement of dihydropteridine reductase activity

Dihydropteridine reductase (DHPR) activity was assayed based on the method of Milstien *et al* (1976). This method monitors the oxidation of the cofactor NADH, in the reduction of quinonoid-dimethyldihydropterin ($q\text{-}DMPH_2$) to dimethyltetrahydropterin ($DMPH_4$), a reaction catalysed by DHPR. The synthetic analogue $DMPH_4$, is used in place of BH_4 due to the instability of BH_4 at room temperature (Howells *et al*, 1986). The reaction scheme for these experiments is shown in figure 2.11. $DMPH_4$ is oxidised by peroxidase, in the presence of H_2O_2 to $q\text{-}DMPH_2$, which is then recycled back to $DMPH_4$ by DHPR, a reaction that utilises NADH. The loss of NADH can be monitored spectrophotometrically at 340nm, and the rate of loss gives an indication of DHPR activity.

Wild type and *hph-1* brain cytosol fractions (section 2.3.1.) were added (20µl volume) to a reaction mixture in a cuvette containing Tris buffer (0.5M Tris HCl pH 6.8), H_2O_2 (8.8mM), peroxidase (25kU/l), NADH (1mM) and 2.5mM sodium azide (to prevent breakdown of H_2O_2 by catalase in cytosol). This cuvette was mixed by inversion, and baseline absorbance of NADH at 340nm was monitored in an Uvikon XL spectrophotometer (Bio-Tek instruments, Vermont, USA), at 37°C for 1.5 minutes. 1mM $DMPH_4$ substrate was then added to make the final reaction mixture volume 1ml, and the change in absorbance was monitored for a further 7 minutes. A graph was plotted of absorbance over time following substrate addition, and the rate of loss of absorbance calculated using the spectrophotometer software. Also included in the experiment was a blank cuvette that did not contain any cytosol, which was subtracted from the rate of change of absorbance in cuvettes with cytosol. Using the Beer-Lambert law with a path length of 1, and molar extinction coefficient for NADH of 6220, the change in concentration of NADH, and thus DHPR activity was

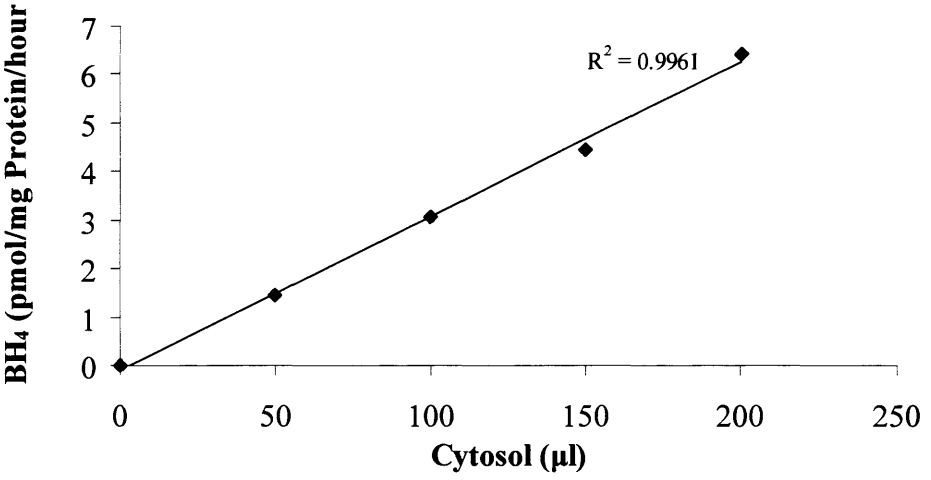


Figure 2.10. BH_4 biosynthetic capacity standard curve with increasing wild type brain cytosol.

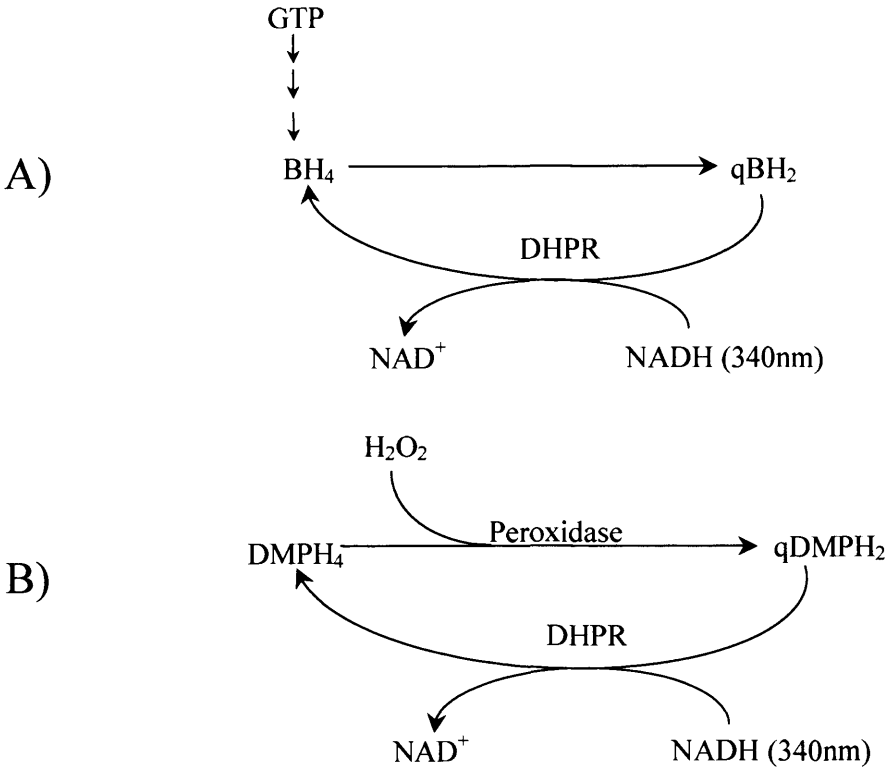


Figure 2.11. Reaction scheme for dihydropteridine reductase assay. A) shows reaction scheme that occurs in the cell, where quinonoid dihydrobiopterin (qBH_2) is converted back to BH_4 by dihydropteridine reductase (DHPR), a reaction that utilises the oxidation of β-nicotinamide adenine dinucleotide (NADH). B) shows the reaction scheme used in the DHPR assay, where dimethyltetrahydropterin ($DMPH_4$) is converted to quinonoid dimethyldihydropterin ($qDMPH_2$) by peroxidase in the presence of hydrogen peroxide (H_2O_2). $qDMPH_2$ is converted back to $DMPH_4$ by DHPR, which causes the loss of NADH, a reaction that can monitored spectrophotometrically at 340nm. $DMPH_4$ is preferred to BH_4 in this assay due to the greater stability of the molecule.

determined. Finally, the values obtained were divided by the protein concentration in cytosol (section 2.9.), and DHPR activity was expressed as nmol/minute/mg protein. To validate this assay a standard curve was produced showing linearity of change in absorbance with increasing cytosol (R^2 0.9859)(Figure 2.12).

2.6. Measurement of nitrite and nitrate

Nitrite and nitrate (NO_3^- and NO_2^- respectively) measurements were made as an index of NO formation (Clelland *et al*, 1996). Quantification of NO_3^- and NO_2^- in cultured cell media was determined spectrophotometrically using the Griess reaction (Green *et al.* 1982) on a 96-well plate (Thermo Labsystems, Vantaa, Finland). Prior to measurement NO_3^- was reduced to NO_2^- by incubation with nitrate reductase (0.1 U/ml)(EC 1.6.6.2)(Roche Diagnostics, Lewes, UK), FAD (5μM) and NADPH (100μM) for 15 minutes at 37°C. Once the NO_3^- reduction is complete, it is necessary to remove any excess NADPH as this can interfere with the spectrophotometric determination of NO_2^-. NADPH is removed in an oxidation reaction with lactate dehydrogenase (100U/ml)(EC 1.1.1.27) and sodium pyruvate (100mM) for 5 minutes at 37°C. Total NO_2^- (representing NO_2^- and reduced NO_3^-) was then measured using the Griess reaction upon the addition of 0.1% napthalethylenediamine dihydrochloride and 1% sulfanilamide/5% H_3PO_4 (Merck Biosciences, Nottingham, UK). This produces a pink diazo product, and the absorbance of this can be read at 540nm using a SPECTRAmax Plus microplate reader (Molecular Devices, Wokingham UK). This method was validated by titrating standard curves of $NaNO_3^-$ and $NaNO_2^-$, which both displayed linearity and >95% conversion of NO_3^- to NO_2^- up to concentrations of 100μM.

2.7. Western blotting

Western blotting is a technique for quantifying specific protein levels in a given sample. This technique entails running a sample through a gel under high electrical current to separate protein by molecular weight – smaller proteins will travel further than large proteins in a given time. These are then transferred to a membrane and probed with antibodies raised against the protein of interest. Bands on an autoradiograph can then be quantified using computer-imaging techniques.

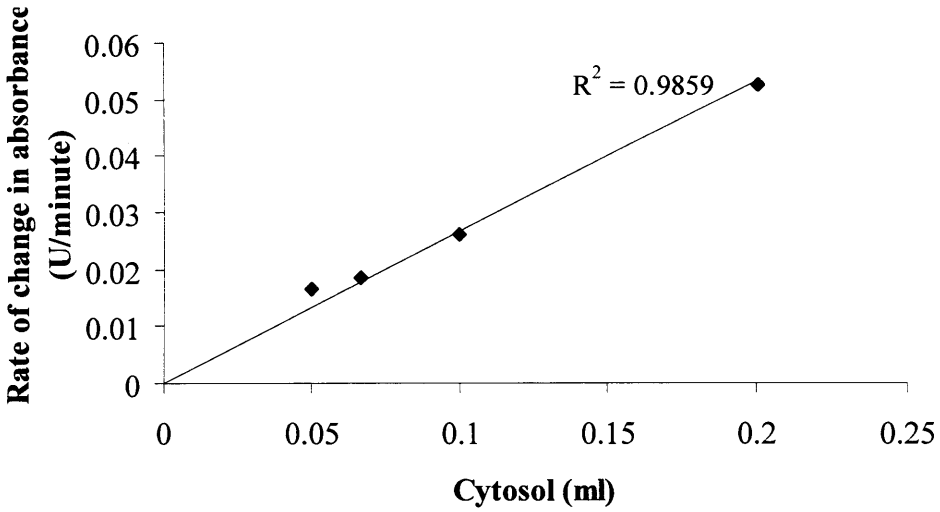


Figure 2.12. Standard curve of DHPR activity (measured by rate of change in absorbance U/minute) with increasing cytosol.

antibodies diluted in 6% Marvel in PBS-T. Details of secondary antibodies used are found in methods section 5.2.5. for each individual protein of interest. Excess washing buffer was removed from the membrane using tissue paper, and membranes were then exposed to Supersignal horseradish peroxidase (HRP) substrate (Pierce, Rockford IL, USA) for 5 minutes. Excess substrate was removed from the membrane using tissue paper, and then exposed to X-ray film (Biomax MR, Eastman Kodak, Rochester, NY) for 1, 10, 20, 60, 120 seconds or 10 minutes, until there was a good contrast between the bands and the background. Films were developed using an Optimax automatic developing machine (IGP, Chelmsford, UK). Bands were quantified using GelPro analysis system and software (Media Cybernetics, Silver Springs, MD) and the density measured in arbitrary units.

2.8. Confocal microscopy of cells infected with AdeGFP

Cells that had taken up the control virus AdeGFP and were expressing eGFP could be visualised using confocal microscopy. Cells were grown to confluence as described in section 2.2.2., and then treated with AdeGFP as described in section 2.2.4. Cells were imaged using a confocal microscope (Bio-Rad MRC1024, Biorad, Hercules, Ca, USA) at an excitation wavelength of 485nm and emission of 550nm.

2.9. Protein analysis

Two different assays were employed to measure protein concentration. In the first protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as a standard. Samples were diluted so the concentration was within the range of the standard curve, in a final volume of 200µl. 100µl of alkaline copper tartrate solution was added to standards and samples, along with 800µl of Folin-Ciocalteau reagent (Bio-Rad). The tubes were vortexed and then incubated in the dark for 20 minutes, before absorbance was measured using an Uvikon XL spectrophotometer (Bio-Tek instruments, Vermont, USA) at 750nm. Sample protein was calculated from the standard calibration curve using regression analysis ($R^2 > 0.95$), with standard protein concentration range varying upon the solution the assay was conducted in. Typical standard concentration ranges for various solutions; H_2O 0 – 0.2mg/ml, 0.5M NaOH 0 - 0.6mg/ml.

A second protein assay was used to calculate protein concentration only for samples used in Western blotting. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. The Bradford assay reagent (Biorad) was diluted 1:5 in 18.2 MΩ Millipore Q H_2O, and 1ml of this mixture was added to a cuvette. 2µl of cell lysate was then added to the reaction mixture, and then mixed by trituration with a 1ml pipette. The absorbance was measured using an Uvikon XL spectrophotometer (Bio-Tek instruments, Vermont, USA) at 595nm. Sample protein was calculated from the standard calibration curve using regression analysis ($R^2 > 0.95$).

2.10. Statistical analysis

In all cases *n* represents independent experiments or individual cell culture and tissue preparations. All results are expressed as mean ± standard error of the mean (SEM). Statistical analysis for single comparisons was made using an unpaired Student's t-test, using Statview statistics package (Statview, Cary, NC, USA). Statistical analysis for multiple comparisons was made by one-way ANOVA, followed by Fischer's least significant difference post-hoc tests also using Statview statistics package. In all cases $p < 0.05$ was considered significant. Ratios of iNOS dimer:monomer formation were transformed through the calculation:

$$\text{Arcsin } \sqrt{} \text{ (dimer expression:monomer expression)}$$

This transformation is reported to yield data with a normal distribution to enable statistical comparisons to be made (Gegg *et al*, 2003).

Chapter 3

Further biochemical characterisation of the hph-1 mouse

3.1 INTRODUCTION

3.1.1 The *hph-1* mouse – historical perspective

The *hph-1* mouse emerged from the effort to develop an animal model of hyperphenylalaninaemia (HPA). One of the first attempts to create such a model was by Cotton in 1986, who wanted to decrease phenylalanine hydroxylase (PAH) activity, and thus induce HPA, by decreasing the availability of the cofactor BH_4 (Cotton, 1986). This was achieved by including the GTP-CH inhibitor diamino-hydroxypyrimidine (DAHP) in the mouse diet, which resulted in undetectable BH_4 levels and increased phenylalanine in serum and liver. However, brain levels of BH_4 did not decrease in adult mice suggesting poor penetration of DAHP across the blood brain barrier, and the study was marked by side effects of the inhibitor such as weight and hair loss, and a death rate of approximately 25% of animals (Cotton, 1986). Therefore this is a relatively poor model of BH_4 deficiency.

An improved animal model for HPA came through the development of a genetic mutant, created using the sperm mutagen N-ethylnitrosourea (ENU). Bode *et al* (1988) used a three-generation breeding scheme in C57Bl/6 x CBA/ca mice to test for recessive mutations that caused HPA in neonates and weanlings (Bode *et al*, 1988). Male mice were administered intraperitoneal injections of ENU, an efficient mutagen of mouse spermatogonial cells (Russell *et al*, 1979), which can produce mutations in specific areas of the mouse genome (Bode *et al*, 1984). These males were then bred with wild type females to produce first generation progeny (G1), heterozygous for mutations at different loci (m_p, m_q, m_r etc.). The G1 male mice were then mated with female wild type mice to produce second-generation progeny (G2). G2 females (m_q/+) were then backcrossed with their G1 m_q/+ fathers, to produce homozygous mutant (m_q/m_q) progeny (G3), which were identified by screening for elevated phenylalanine. The diagram in figure 3.1 illustrates this process. These mice were named *hph* due to the HPA and given the suffix 1, as they were the first of several HPA lines produced.

3.1.2 *hph-1* phenotype

In general *hph-1* mice are smaller in size and weight than their wild type counterparts, have lower brain weight and produce litters that are smaller in size

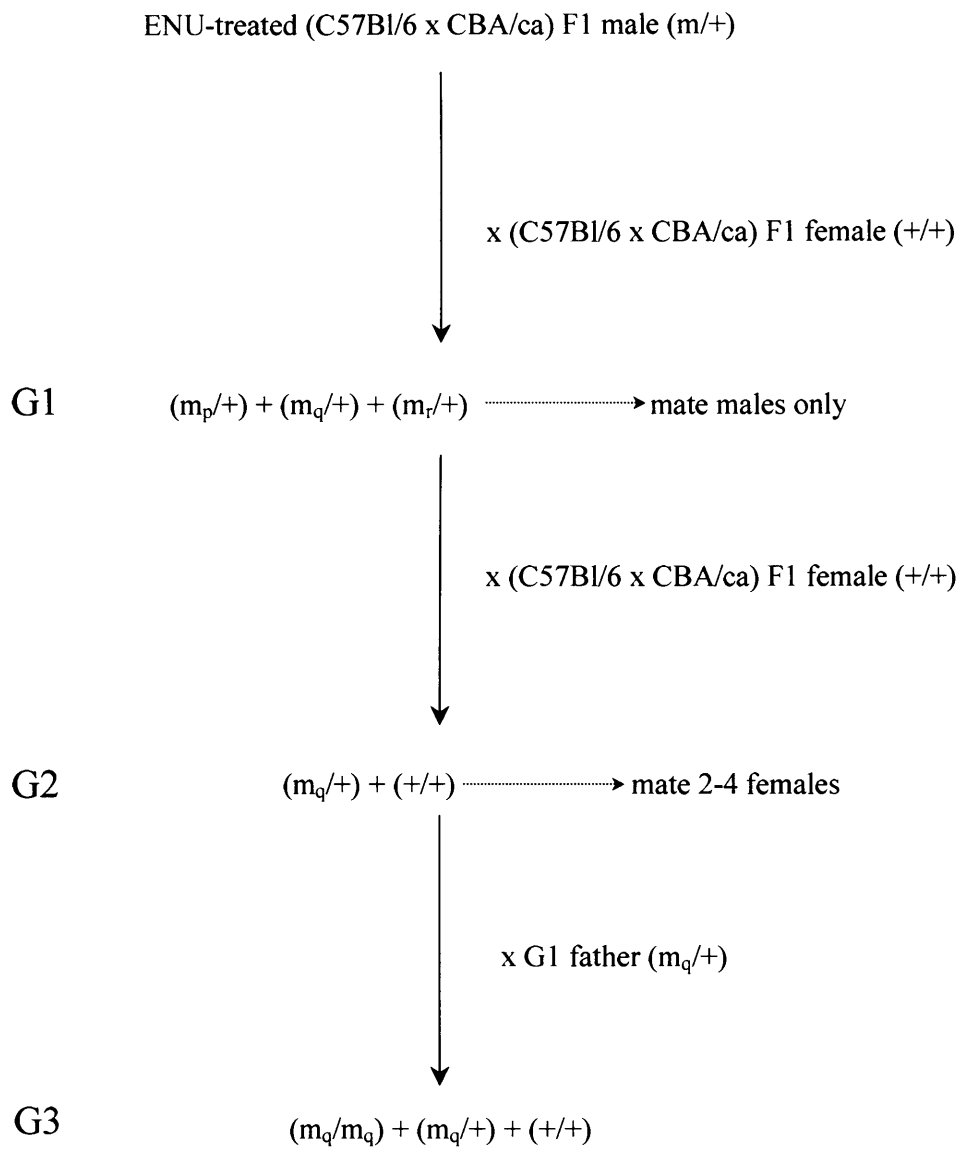


Figure 3.1. An outline of the three-generation ENU-mutagenesis scheme used to produce homozygous mutant *hph-1* mice.

G1, G2 and G3 are first, second and third generations, respectively. (+/+) are wild type mice, (m_p/+), (m_q/+), (m_r/+) are carriers of mutations at gene p, q and r, respectively. (m_q/m_q) are homozygous for mutation in gene q. See text for further details. Adapted from Bode *et al*, 1988.

(Brand, 1997). However, so far researchers have not conducted a full investigation of the phenotype of the *hph-1* mouse, particularly with regard to any possible movement disorder the mouse might exhibit.

3.1.3 *hph-1* biochemical phenotype

3.1.3.1. *Phenylalanine metabolism*

The *hph-1* mouse displays HPA at birth, which is eventually normalised after the first 3 weeks of its life (Bode *et al*, 1988; McDonald and Bode, 1988). This reduction in plasma phenylalanine correlates with the slow accumulation of BH_4 and increase of GTP-CH activity in the liver of these mice over time, up to approximately 50% of wild type (Gütlich *et al*, 1994; Hyland *et al*, 1996). However, if the mice are challenged with a bolus of phenylalanine, the metabolism of phenylalanine to tyrosine remains compromised, as levels in plasma of phenylalanine increase dramatically for a prolonged period. After phenylalanine challenge tyrosine is not raised in *hph-1* mice as it is in wild type, which reflects their impaired ability to convert phenylalanine to tyrosine (McDonald and Bode, 1988).

3.1.3.2 *Tetrahydrobiopterin metabolism*

GTP-CH activity is impaired in the brain and liver of the *hph-1* mouse (McDonald *et al*, 1988), which results in lower BH_4 levels in these tissues (Hyland *et al*, 1996). In the liver the mutant enzyme shows only 8% of normal activity compared to wild type (Cha *et al*, 1991). The specific mutation that causes the *hph-1* phenotype is currently unknown. Initial work confirmed that the mutation is close to the *GCH* gene encoding GTP-CH (Montañez and McDonald, 1999), and more recent evidence localises the *hph-1* mutation to a congenic interval of 1.6-2.8 Megabases, a region that contains *GCH* (Khoo *et al*, 2004). Sequencing of the coding and 5' flanking promoter of the *GCH* gene shows that the mutation is not found in either of these regions (Gütlich *et al*, 1994; Shimoji *et al*, 1999; Maeda *et al*, 2000), and thus the mutation is unlikely to affect the enzyme directly. These data are confirmed by experiments expressing *hph-1* GTP-CH enzyme in *E-Coli* that reported normal enzyme activity (Gütlich *et al*, 1994). Northern blotting shows that the steady state level of GTP-CH mRNA are decreased in the *hph-1*, so Gütlich *et al* suggest the mutation may affect the binding of transcription factors to the promoter leading to decreases in mRNA (Gütlich *et al*, 1994).

3.1.3.3. *Monoamine metabolism*

Decreased BH_4 levels result in lower TH and TRH activities, and reduced TH expression in the brain of the *hph-1* mouse. This gives rise to lower levels of 5-HT, DA and NA, plus their metabolites 5-HIAA, HVA and MHPG (Hyland *et al*, 1996). Unlike the HPA these animals experience, decreased monoamines persist into adulthood (Hyland *et al*, 1996). Administration of acute non-toxic doses of BH_4 resulted in an increase in brain BH_4 that subsided after 24 hours, however this was not accompanied by increases in TH or TRH activity or monoamine concentrations (Brand *et al*, 1996). Only toxic doses (1mmol/kg) of BH_4 could elevate DA or 5-HT turnover, which lasted 24 hours. It is suggested that BH_4 may control the *de novo* synthesis of the aromatic amino acid monooxygenases, or may be required for their stability, as decreased expression of PAH and TH are found in the *hph-1* mouse (Hyland et al, 1996). This may be why the non-toxic acute doses of BH_4 administered were not sufficient to increase activity (Brand *et al*, 1996). Consequently, a more chronic dosing regimen may be necessary to correct brain monoamine neurotransmitter levels in the *hph-1*.

3.1.3.4. *Nitric oxide metabolism*

In contrast with the aromatic amino acid mono-oxygenases, NOS activity is comparable to wild type in the *hph-1* brain (Brand *et al*, 1995). However, when exogenous BH_4 is left out of the NOS assay, a 20% loss of activity was observed in *hph-1* brain cytosol only (Brand *et al*, 1995). In addition, the K_m of NOS for the substrate arginine is significantly higher in *hph-1* brain preparations in the absence of exogenous BH_4, suggesting that less substrate will bind to *hph-1* NOS in the brain and generate less NO (Brand *et al*, 1995). These decreases in NOS activity and arginine binding result in decreased amounts of cGMP, the downstream messenger of NO signalling, in *hph-1* cerebellar slices and homogenates (Brand *et al*, 1996; Canevari *et al*, 1999). This can be corrected by the addition of an NO donor (Brand *et al*, 1996) or by intraperitoneal injections of BH_4 (Canevari *et al*, 1999). In astrocytes cultured from *hph-1* mouse brain, stimulation of iNOS by LPS plus IFN-γ induces increases in BH_4 and NO, in both wild type and *hph-1* cells (Barker *et al*, 1998). However the increases were significantly attenuated in *hph-1* astrocytes, even though iNOS specific activity and expression were found to be elevated under these conditions (Barker *et al*, 1998). This is suggested to occur because BH_4 does not

regulate the intracellular concentration of NOS protein (Brand *et al*, 1995), and NO may act via negative feedback mechanisms to control iNOS expression (Barker *et al*, 1998). Thus, decreased production of NO results in less inhibition of iNOS and therefore greater activity and expression in *hph-1* astrocytes.

As well as having a deficiency in the brain, *hph-1* mice also display impaired NO metabolism in the periphery. Most of the effects seen outside the CNS are related to the role of BH_4 as a cofactor for NOS. For example, in aortas taken from *hph-1* and wild type mice, eNOS activity in the absence of exogenous BH_4 is decreased in the *hph-1* mouse (Cosentino *et al* 2001). Furthermore, lower tissue levels of BH_4 are associated with decreased nitrite and nitrate levels, and increased $\cdot O_2^-$ production in the periphery (Cosentino *et al*, 2001; Nandi *et al*, 2005). In addition, increased H_2O_2 production is reported in *hph-1* aortic segments, which can mediate endothelium-dependent relaxations to acetylcholine, suggesting that at sub-optimal BH_4 conditions reactive oxygen species (ROS) are produced by NOS (Cosentino *et al*, 2001). Uncoupling of the NOS reaction and (ROS) generation may explain the increased systolic blood pressure (Cosentino *et al*, 2001) and pulmonary but not systemic hypertension (Nandi *et al*, 2005; Khoo *et al*, 2005), as well as exacerbated neointimal formation after vascular injury (Wang *et al*, 2005), that are features of the *hph-1* pathophysiology. Furthermore, crossing the *hph-1* mouse with a mouse that over-expresses GTP-CH can rescue the pulmonary hypertensive state (Khoo *et al*, 2005), which further suggests that the impaired NO metabolism in the periphery is related to GTP-CH deficiency in the *hph-1* mouse.

3.1.4 *hph-1* and dopa responsive dystonia

Biochemically, dopa responsive dystonia (DRD) (section 1.11.5) and the *hph-1* mouse appear to have much in common. The similarities include first and foremost a partial BH_4 deficiency resulting from a mutation relating to the GTP-CH enzyme; as well as decreases in DA metabolism, and impaired phenylalanine clearance when responding to a phenylalanine load (Hyland *et al*, 2003). Because of the biochemical similarities between DRD and the *hph-1* mouse, it has been considered that the latter may be a good model for the former (Hyland *et al*, 2003).

This chapter will further characterise the BH_4 and monoamine deficiencies in the *hph-1* mouse and investigate the effect of the mutation on the activity of the biosynthetic and recycling pathways.

3.2. AIMS

- Further establish the deficiency of BH_4 in the brain of the *hph-1* mouse.
- Determine the ability of the *hph-1* mouse to generate BH_4 from the initial substrate GTP, by measuring the biosynthetic capacity of the *de novo* synthesis pathway.
- Investigate whether the recycling pathway is intact in the *hph-1* mouse by measuring dihydropteridine reductase activity, i.e. to show BH_4 can be recycled if gene transfer is used to increase levels of the cofactor.
- Bypass the *hph-1* metabolic block in *hph-1* brain and measure the ability of the enzymes downstream of GTP-CH to produce BH_4, i.e. to illustrate that if gene transfer is used to increase GTP-CH expression, the rest of the pathway is intact to synthesize normal levels of BH_4.
- Further establish the monoamine deficiency in the brain of the *hph-1* mouse to show that impaired BH_4 leads to decreased levels of monoamine neurotransmitters, with an ultimate view to correcting this using gene transfer.

3.3. METHODS

3.3.1. Tetrahydrobiopterin measurement

Brain samples were prepared as described in section 2.3.1. BH_4 was measured by reverse-phase HPLC, as described in section 2.1.

3.3.2 Measurement of monoamine neurotransmitters and metabolites

Brain samples were prepared as described in section 2.3.1. The monoamine neurotransmitters and their metabolites were measured by reverse-phase HPLC, as described in section 2.1.

3.3.3. Tetrahydrobiopterin biosynthetic capacity measurements

A description of the BH_4 biosynthetic capacity assay is found in section 2.4.

3.3.4. Dihydropteridine reductase assay

DHPR activity was measured spectrophotometrically as described in section 2.5.

3.3.5. Biosynthetic capacity bypass block experiments

As dihydroneopterin triphosphate (DHNTP), the product of GTP-CH and substrate for the next enzyme in the pathway PTPS, is not commercially available it was not possible to purchase this to bypass the block. Therefore it was necessary to synthesize DHNTP via recombinant human GTP-CH (kindly provided by Gabrielle Werner-Felmayer, Innsbruck Medical University, Innsbruck, Austria). DHNTP was synthesized according to the method of Werner *et al*, 1997. Recombinant human GTP-CH purified from Escherichia coli (4×10^{-3} Units/ml final concentration) was incubated with 100mM Tris HCl pH 7.6, 100mM KCl, 10mM EDTA, and 100μM or 40μM GTP (final reaction volume 250μl). This solution was incubated at 37°C in the dark for 1 hour, before the reaction was terminated by centrifuging the solution at 15,000 x g for 15 minutes at 4°C, through Ultrafree-MC filters with a molecular weight cut off of 10kDa (Millipore, Bedford, MA, USA). This was to remove any recombinant human GTP-CH that may produce DHNTP in the next reaction solution, as the approximate molecular weight of the individual GTP-CH subunits is 30kDa (Togari *et al*, 1992). This solution was then added in place of GTP to the biosynthetic capacity assay, which was carried out as previously described (section

2.4). The final concentrations of DHNTP in the biosynthetic capacity assay were 8μM and 20μM (assuming all GTP was converted in the DHNTP preparation). The reaction was terminated after 3 hours according to the method of Barford *et al*, 1984, using 100mM PCA buffer containing 6.5mM DTE and 2.5mM DETAPAC, and BH_4 was measured as described in section 2.1. As controls an inhibitor of GTP-CH diamino-6-hydroxypyrimidine (DAHP; 5.0mM), and the equivalent concentrations of 8μM and 20μM GTP were included in these experiments, in case any GTP was not converted to DHNTP in the initial preparation. Inclusion of GTP also allows the comparison of biosynthetic capacity pre- and post-GTP-CH.

The final concentrations of DHNTP and GTP added to cytosolic fractions in this assay are lower than the concentrations of GTP used in the initial biosynthetic capacity assay described above in section 2.4. In the initial assay the final concentration of GTP used is 6mM, whereas only 8μM and 20μM DHNTP and GTP are incubated with brain cytosol fractions in bypass block experiments. This is because the assay described by Werner *et al*, 1997 to make DHNTP, uses lower concentrations of GTP in the initial incubation, which is diluted further when added to brain cytosol. This may explain discrepancies between values of biosynthetic capacity between the two experiments.

3.3.6. Protein concentration

The protein concentration of the cytosol used in biosynthetic capacity and dihydropteridine reductase assays was measured by the Lowry method (1951) as described in section 2.9. Protein concentrations for cytosol were calculated based on a standard curve of bovine serum albumin dissolved in 0.1 M Tris HCl pH 7.6, with concentrations of 0.005 mg/ml to 0.2 mg/ml.

3.3.7. Statistical analysis

Statistical tests were conducted as described in section 2.10.

3.4. RESULTS

3.4.1. Tetrahydrobiopterin levels in whole brain of wild type and *hph-1* mice

BH_4 levels were significantly lower ($p < 0.001$, $n = 3$) in the whole brain of *hph-1* mice when compared to the wild type mice (figure 3.2). BH_4 levels in the *hph-1* mouse brain (104.43 ± 3.64 pmol/ g wet weight tissue) were 31% of wild type (332.63 ± 11.97 pmol/ g wet weight tissue).

3.4.2. Tetrahydrobiopterin biosynthetic capacity in whole brain tissue of wild type and *hph-1* mice

BH_4 biosynthetic capacity was used to give a comparison of the activity of the BH_4 *de novo* biosynthesis pathway in *hph-1* and wild type mice, to investigate whether the proposed defect in the *hph-1* mouse affects its ability to synthesize BH_4 from the initial substrate GTP. BH_4 biosynthetic capacity in the *hph-1* mouse brain was significantly lower ($p < 0.05$, $n = 3$) than its wild type counterpart. Mean values for the *hph-1* biosynthetic capacity were 1.01 ± 0.02 pmol/mg protein/hour, against wild type values of 2.49 ± 0.34 pmol/mg protein/hour, a decrease of 59% (figure 3.3).

3.4.3. Dihydropteridine reductase activity in whole brain tissue of wild type and *hph-1* mice

Dihydropteridine reductase (DHPR) activity gives a measure of the activity of the recycling pathway, which regenerates BH_4 once it has been used in its cofactor role (see section 1.4). DHPR activity was comparable in wild type and *hph-1* whole brain cytosol fractions ($p = 0.435$, $n = 6$). Mean ± SEM values for wild type DHPR activity were 219.33 ± 14.74nmol/minute/mg protein, versus 201.67 ± 15.97nmol/minute/mg protein (figure 3.4.) for *hph-1*.

3.4.4. Comparison of the biosynthetic capacity of the enzymes downstream of GTP cyclohydrolase 1 in wild type and *hph-1* brain

These experiments were designed to introduce dihydroneopterin triphosphate (DHNTP) downstream of GTP-CH in the BH_4 biosynthetic pathway, and then compare the abilities of wild type and *hph-1* brain cytosolic fractions to produce BH_4. However, before this comparison was carried out some experiments were

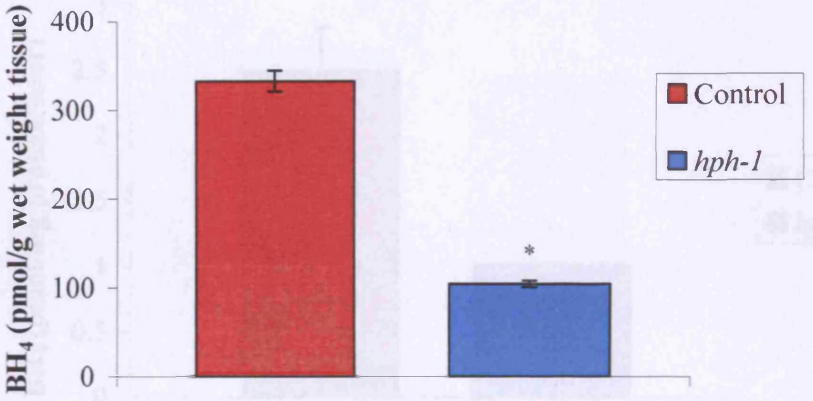


Figure 3.2. BH_4 levels in whole brain homogenates of *hph-1* and wild type mice. BH_4 was measured by reverse-phase HPLC coupled with ECD. Data is expressed as mean ± SEM (n = 3). * = $p < 0.0001$ vs. wild-type, as judged using Student's t-test.

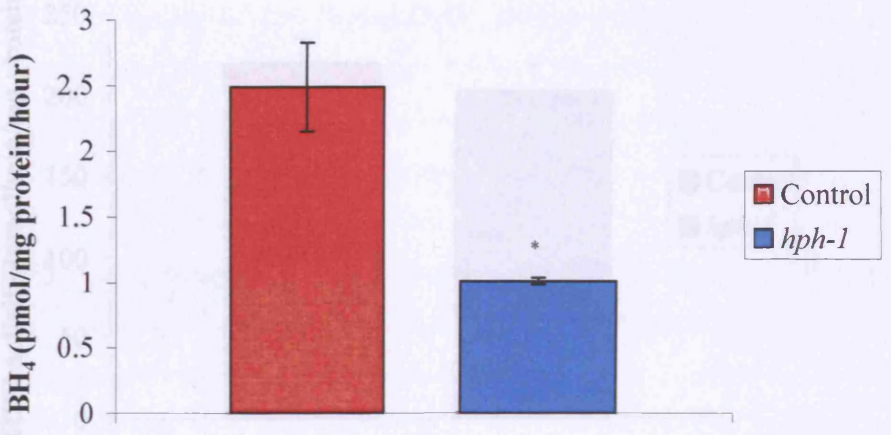


Figure 3.3. Comparison of BH_4 biosynthetic capacity levels using whole brain cytosol fractions of *hph-1* and wild type mice. See section 2.4. for assay details. Data is expressed as mean ± SEM (n = 3). * = $p < 0.05$ vs. wild-type, as judged using Student's t-test.

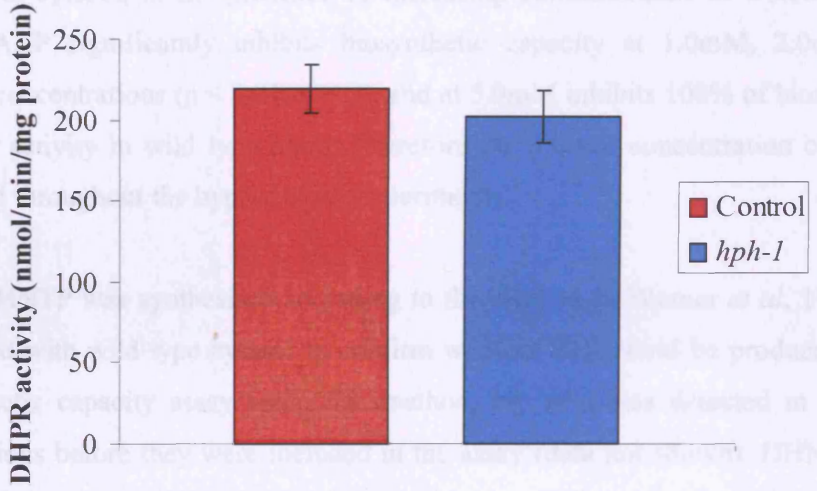


Figure 3.4. Comparison of dihydropteridine reductase (DHPR) activity levels in cytosol of *hph-1* and wild type mice. See section 2.5. for assay details.
BH_4 was measured by reverse-phase HPLC coupled with ECD. Data is expressed as mean ± SEM (n = 6).

conducted to validate this approach. Diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP-CH is included in some of the incubations, as a control measure to show that BH_4 produced from DHNTP is not from uncatalysed GTP left in the preparation. To begin with, it was necessary to find the appropriate concentration of DAHP to use, so the biosynthetic capacity assay (section 2.4.) was carried out in wild type brain cytosol, in the presence of increasing concentrations of DAHP (figure 3.5). DAHP significantly inhibits biosynthetic capacity at 1.0mM, 2.0mM and 5.0mM concentrations ($p < 0.01$, $n = 3$), and at 5.0mM inhibits 100% of biosynthetic capacity activity in wild type brain. Therefore the 5.0mM concentration of DAHP was used throughout the bypass block experiments.

Next, DHNTP was synthesised according to the method of Werner *et al*, 1997, and incubated with wild type cytosol to confirm whether BH_4 could be produced in the biosynthetic capacity assay using this method. No BH_4 was detected in DHNTP preparations before they were included in the assay (data not shown). DHNTP lead to significant increases in biosynthetic capacity in wild type brain cytosol, at both 8μM and 20μM concentrations (figure 3.6.). Furthermore, as the theoretical concentration of DHNTP is augmented from 8μM to 20μM, biosynthetic capacity significantly increases ($p < 0.05$, $n = 3$). However, when DAHP is included in the incubation, it significantly decreases BH_4 formation by 76% at 8μM ($p < 0.01$, $n = 3$) and 78% with 20μM DHNTP ($p < 0.01$, $n = 3$)(figure 3.6). This suggests that uncatalysed GTP may be responsible for a large part of the biosynthetic capacity from the DHNTP preparation.

However, when the theoretically comparable concentrations of GTP (8μM and 20μM) were substituted in place of DHNTP in this assay, biosynthetic capacity was significantly decreased using GTP when compared to the DHNTP incubations, even in the presence of DAHP (Table 3.1). Furthermore, biosynthetic capacity using 8μM and 20μM GTP did not increase biosynthetic capacity above blank levels (table 3.1). This suggests that DHNTP hypothetically produced using this assay, may be the source of the increased biosynthetic capacity in figure 3.6 and can be used to bypass the metabolic block in *hph-1* brain cytosol fractions. In addition, the rate of BH_4 biosynthetic capacity is much greater post-GTP-CH, implying that GTP-CH is the rate-limiting step in the pathway.

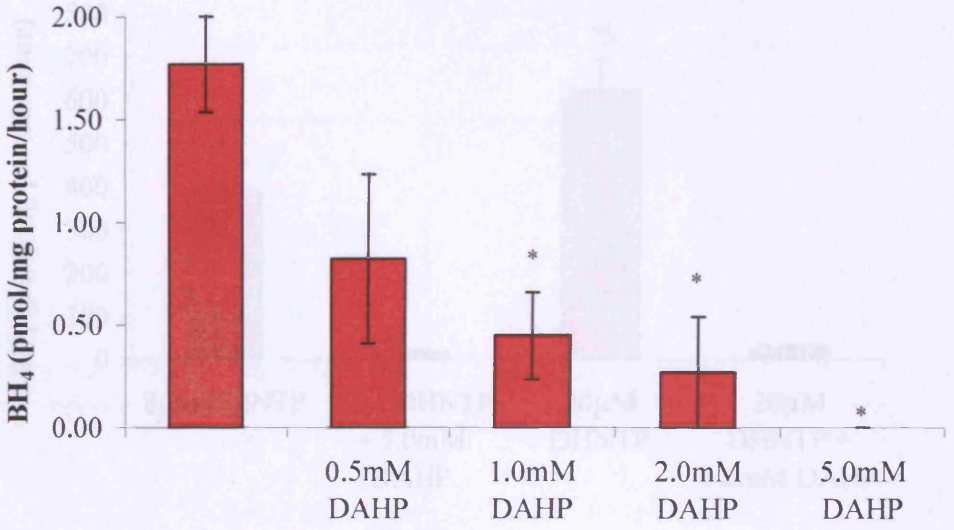


Figure 3.5. BH_4 biosynthetic capacity assay in the presence and absence of increasing concentrations of 2,4-diamino-6-hydroxypyrimidine (DAHP), in whole brain homogenates of wild type mice. Biosynthetic capacity was measured using 6mM guanosine 5'- triphosphate (GTP) incubated with cytosol from whole brain, and then inhibited with concentrations of DAHP ranging from 0.5mM to 5.0mM.
Data is expressed as mean ± SEM of separate mouse brain cytosol fractions (n = 3).
*, $p < 0.01$ vs. stimulated alone, as judged using Student's t-test.

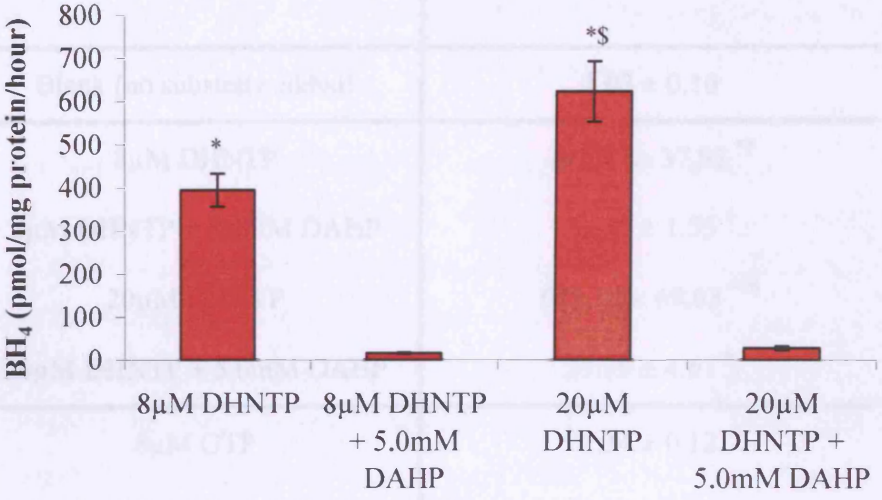


Figure 3.6. Biosynthetic capacity in wild type brain cytosol fractions, incubated with dihydroneopterin triphosphate (DHNTP) in the presence and absence of 5.0mM diamino-6-hydroxypyrimidine (DAHP). Data expressed as mean ± SEM of 3 independent brain cytosol biosynthetic capacity experiments. *, $p < 0.01$ vs. 5.0mM DAHP treated biosynthetic capacity. $^{\$}$, $p < 0.05$ vs. 8μM DHNTP. Statistics calculated using one way ANOVA followed by least significant difference test See section 3.2.5. for details of assay and DHNTP preparation.

Treatment	BH_4 pmol/mg protein/hour
Blank (no substrate added)	1.03 ± 0.16
8μM DHNTP	394.99 ± 37.92 *†
8μM DHNTP + 5.0mM DAHP	15.82 ± 1.55 †
20μM DHNTP	623.34 ± 69.63 *†$
20μM DHNTP + 5.0mM DAHP	27.79 ± 4.67 †
8μM GTP	1.34 ± 0.12
8μM GTP + 5.0mM DAHP	0.89 ± 0.26
20μM GTP	1.12 ± 0.34
20μM GTP + 5.0mM DAHP	1.12 ± 0.62

Table 3.1. Effect of different concentrations of guanosine 5'-triphosphate (GTP) and dihydroneopterin triphosphate preparation (DHNTP), on biosynthetic capacity in wild type brain cytosolic fractions, in the presence and absence of 5.0mM diamino-6-hydroxypyrimidine (DAHP). Amounts of GTP and DHNTP given as final concentrations in the biosynthetic capacity assay. Data expressed as mean ± SEM of 3 independent brain cytosol biosynthetic capacity experiments. *, $p < 0.01$ vs. GTP biosynthetic capacity. †, $p < 0.01$ vs. 5.0mM DAHP treated biosynthetic capacity. $, $p < 0.05$ vs. 8μM DHNTP. Statistics calculated using one way ANOVA followed by least significant difference test. See section 3.2.5. for details of assay and DHNTP preparation.

Finally, biosynthetic capacity was then compared between wild type and *hph-1* cytosol incubated with the 8μM DHNTP preparation, in the presence of DAHP (figure 3.7). The biosynthetic capacity in wild type cytosol (12.48 ± 2.31 pmol/mg protein/hour; n = 6) was not significantly different from *hph-1* cytosol (11.62 ± 1.44 pmol/mg protein/hour; n = 6) incubated with DHNTP, suggesting that the enzymes downstream of the GTP-CH metabolic block function normally in the *hph-1* mouse.

3.4.5. Monoamine levels in whole brain of wild type and *hph-1* mice

Concentrations of NA, dopamine DA, the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) and a metabolite of DA, homovanillic acid (HVA), were measured in the brains of 30-day-old wild type and *hph-1* mice. Although it was possible to measure standards of Dopac and MHPG, metabolites of DA and NA respectively, in brain tissue these peaks were obscured by the solvent front under the chromatographic conditions employed here. Levels of NA were found to be significantly lower in *hph-1* brains in comparison to wild type (1454 ± 99 vs. 1882 ± 226 pmol/ g wet weight tissue; $p < 0.05$, n = 6), a decrease of approximately 23% (figure 3.8). Furthermore, in *hph-1* brain tissue concentrations of DA (3091 ± 235 vs. 3612 ± 261 pmol/ g wet weight tissue; $p < 0.05$, n = 6) and HVA (409 ± 52 vs. 679 ± 74 pmol/ g wet weight tissue; $p < 0.05$, n = 5) were decreased by 14% and 40%, respectively. In addition, 5-HIAA levels were diminished by approximately 55% in *hph-1* mouse brains (331 ± 58 vs. 736 ± 86 pmol/ g wet weight tissue; $p < 0.05$, n = 6).

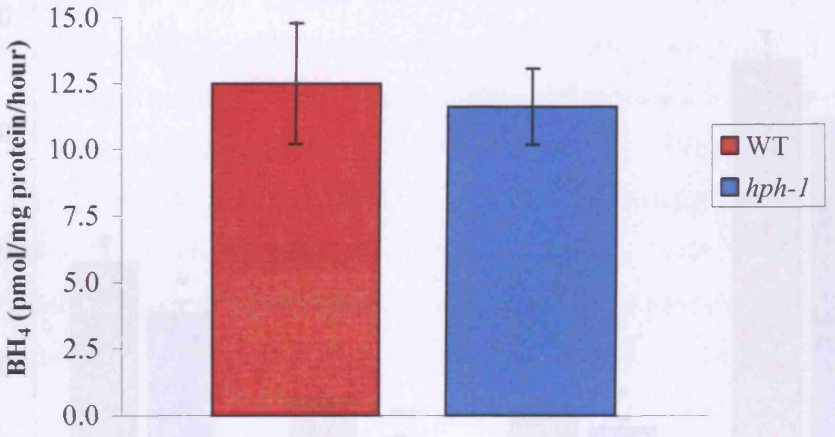


Figure 3.7. Comparison of BH_4 biosynthetic capacity levels using 8 µM dihydroneopterin triphosphate preparations in the presence of 5.0mM diamino-6-hydroxypyrimidine (DAHP), in whole brain cytosol fractions of *hph-1* and wild type mice.

BH_4 was measured by reverse-phase HPLC coupled with ECD. Data expressed as mean ± SEM of 6 independent brain cytosol biosynthetic capacity experiments. See section 3.2.5. for details of assay and DHNTP preparation.

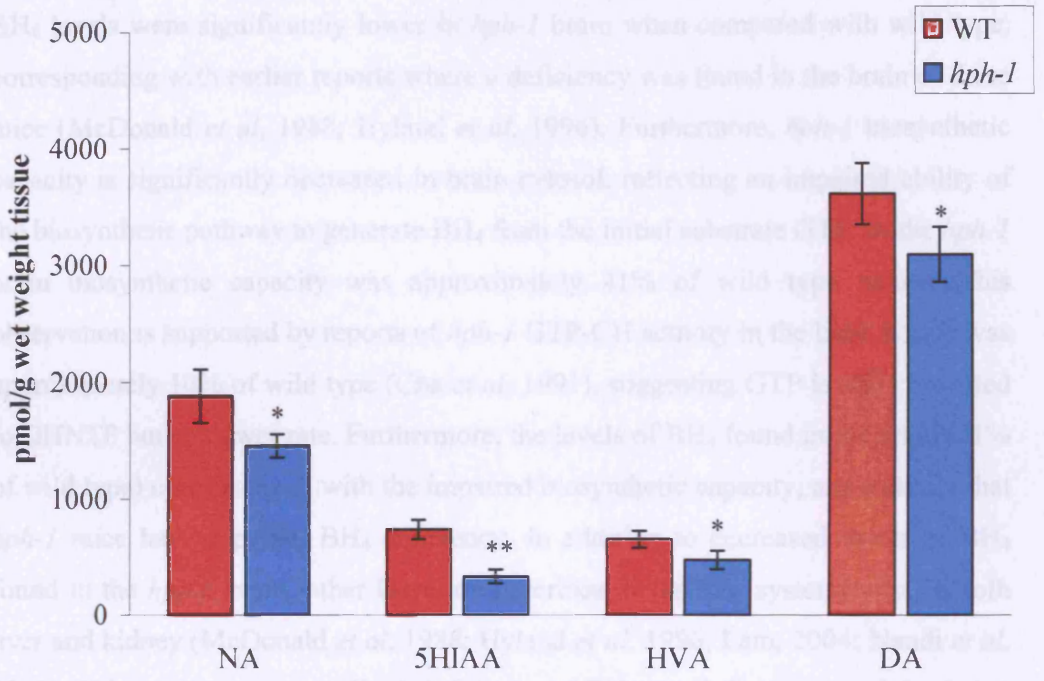


Figure 3.8. Comparison of monoamine neurotransmitters plus their metabolite levels, in whole brain homogenates of *hph-1* versus wild type 30-day-old mice. Noradrenaline (NA), dopamine (DA), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were measured by reverse-phase HPLC coupled with coulometric detection. Data is expressed as mean ± SEM (n = 6-7). * = $p < 0.05$ vs. wild-type, ** = $p < 0.01$ vs. wild-type, as judged using Student's t-test.

3.5. DISCUSSION

3.5.1. Tetrahydrobiopterin deficiency in the *hph-1* mouse brain

BH_4 levels were significantly lower in *hph-1* brain when compared with wild type, corresponding with earlier reports where a deficiency was found in the brain of these mice (McDonald *et al*, 1988; Hyland *et al*, 1996). Furthermore, *hph-1* biosynthetic capacity is significantly decreased in brain cytosol, reflecting an impaired ability of the biosynthetic pathway to generate BH_4 from the initial substrate GTP. In the *hph-1* brain biosynthetic capacity was approximately 41% of wild type values. This observation is supported by reports of *hph-1* GTP-CH activity in the liver, which was approximately 10% of wild type (Cha *et al*, 1991), suggesting GTP is still converted to DHNTP but at slower rate. Furthermore, the levels of BH_4 found in the brain (31% of wild type) correlate well with the impaired biosynthetic capacity, and indicate that *hph-1* mice have a partial BH_4 deficiency. In addition to decreased levels of BH_4 found in the *hph-1* brain, other literature describes lower BH_4 systemically, in both liver and kidney (McDonald *et al*, 1988; Hyland *et al*, 1996; Lam, 2004; Nandi *et al*, 2005). This suggests a generalised deficiency of BH_4 in all the tissues of the *hph-1* mouse.

3.5.2. Recycling pathway in the *hph-1* mouse brain

The BH_4 recycling pathway regenerates BH_4 once it has been used in its cofactor role (Kaufman, 1964)(section 1.4.), and the integrity of this pathway can be measured by assaying the activity of DHPR. It was found that the recycling pathway was not significantly impaired in *hph-1* brain cytosol fractions, indicating that it does not contribute to the BH_4 deficiency observed in the *hph-1* mouse brain. BH_4 levels in CSF and urine from patients with DHPR deficiency appear to be within the normal range in these individuals, suggesting that a mutation giving rise to impaired DHPR may not actually lead to brain BH_4 deficiency in any case (Hyland and Heales, 1993; Ponzone *et al*, 2004). These data in the *hph-1* brain indicate that if gene therapy is used to correct the GTP-CH deficiency, an intact recycling pathway exists to maintain normal BH_4 levels in these mice.

3.5.3. Comparison of the biosynthetic capacity of the enzymes downstream of GTP cyclohydrolase 1, in wild type and *hph-1* brain

Incubation of "DHNTP" preparations with brain cytosol produced large concentration-dependent increases in the biosynthetic capacity that were several hundred-fold greater than capacities measured using the same theoretical corresponding concentrations of GTP. This suggests that GTP-CH is indeed the rate-limiting step in the pathway in this tissue. A study measuring the activities of the BH_4 biosynthetic enzymes in human macrophages, fibroblasts, THP-1 and T24 cells shows that under basal conditions PTPS and SR activities are a minimum of 10-fold higher than GTP-CH (Werner *et al*, 1990). Furthermore, PTPS and SR do not appear to be under the control of negative feedback, unlike GTP-CH with GFRP. Therefore a substrate introduced downstream of GTP-CH could be converted to BH_4 more rapidly and in greater amounts, which appears to be the case in these experiments.

DAHP, an inhibitor of GTP-CH, was included as a control in these experiments, along with the equivalent theoretical concentrations of GTP, to show that any BH_4 produced from DHNTP was not from uncatalysed GTP in the initial preparation. Biosynthetic capacity using GTP was negligible compared to the rate using DHNTP, suggesting DHNTP is indeed the source of biosynthetic capacity. However, DAHP was shown to inhibit DHNTP-stimulated biosynthetic capacity by approximately 75%. DAHP is reported to be a selective and direct acting inhibitor of GTP-CH (Xie *et al*, 1998), although recent evidence now suggests that DAHP is only a weak competitive inhibitor of GTP-CH, and mainly exerts its action through GTP-CH feedback regulatory protein (GFRP)(Kolinsky and Gross, 2004). DAHP is only effective at inhibiting GTP-CH at millimolar concentrations (Xie *et al*, 1998; Kolinsky and Gross, 2004), which suggests that non-specific inhibitory actions could be responsible for the decrease in DHNTP-stimulated biosynthetic capacity seen here. However, no data is currently published concerning the effects of DAHP on the activities of the other enzymes in the biosynthetic pathway, which could be potential future work.

Despite the reservations over DAHP as a specific inhibitor of GTP-CH, the subtracted DAHP rate was used to compare biosynthetic capacity between wild type and *hph-1* cytosolic fractions. A comparison of the capacities of the enzymes

downstream of GTP-CH, revealed no difference between wild type and *hph-1* mice. This would suggest that PTPS and SR may be unaffected by the metabolic block in the *hph-1* mice. Therefore, if gene transfer were used to attempt to alleviate the *hph-1* defect by transfecting these animals with GTP-CH, the rest of the biosynthetic pathway may be integral to produce BH_4.

There is some evidence of the enzymes downstream of GTP-CH being modulated as the levels or activity of GTP-CH changes. Studies in human neuroblastoma cells treated with α-synuclein show that down-regulation of GTP-CH expression leads to a decrease in SR, but not PTPS expression (Baptista *et al*, 2003). α-synuclein is linked to dopaminergic cell death in Parkinson's disease, and cells treated with this had lower levels of both GTP-CH and SR mRNA and protein. Moreover, GTP-CH, PTPS and SR activities have all been shown to increase in parallel in human neuroblastoma cells, in response to LPS stimulation (Mori *et al*, 1997).

Studies in macrophages, THP-1, fibroblasts and T24 cells report PTPS and SR activities to vary from approximately 10 – 25,000 fold higher than GTP-CH (Werner *et al*, 1990). Stimulation of these cells with the cytokine IFN-γ, results in large changes in GTP-CH activity, however PTPS or SR activities are not altered (Werner *et al*, 1990). So therefore unless the expression of these two enzymes is dramatically altered by the amount of GTP-CH present, there are unlikely to be large changes in the biosynthetic capacity of the BH_4 biosynthesis pathway. In patients with the autosomal dominant form of GTP-CH deficiency (DRD), levels of GTP-CH activity and expression are decreased, however SR activity appears to be within the normal range (Blau *et al*, 2001b). These data in DRD patients appear to be in consensus with the results reported here, suggesting no change in the activity of the enzymes downstream of GTP-CH in the *hph-1* mouse.

3.5.4. Monoamine deficiency in the *hph-1* mouse

Analysis of levels of DA, NA, HVA and 5-HIAA revealed significant deficiencies of all the neurotransmitters and their metabolites in the *hph-1* brain in comparison to their wild type counterparts. These data are supported by earlier reports of Hyland *et al*, (1996), who examined monoamine concentrations in *hph-1* and wild type mice, and found deficiencies in all of the above, plus 5-HT, Dopac and MHPG, metabolites

of DA and NA, respectively. Unfortunately, it was not possible to optimise conditions to allow us to measure all these seven neurotransmitters and their metabolites, but the four analysed give us a good index of NA, DA and 5-HT status and turnover in the *hph-1* brain. The concentrations and percentage changes of neurotransmitters and their metabolites reported here are similar to published values, (Hyland *et al*, 1996), although the absolute values are in the range of 30-50% lower. This may be explained by differences in diet or breeding conditions, between this study and Hyland *et al* (1996), or differences in the sensitivity between the two assays, but not age as in both experiments mice were approximately 30 days old.

Many factors may be behind the difference in DA, NA and 5-HT levels found in *hph-1* mice. Shimoji *et al*, 1999 found that GTP-CH mRNA levels were significantly lower than wild type in *hph-1* in the CNS, and lowest in serotonergic, followed by dopaminergic then noradrenergic neurones. This may account for why 5-HIAA production appears to be most strongly affected by the *hph-1* genotype, although it is difficult to say given this evidence alone. Hyland *et al* (1996) showed lower activities of TH and TRH enzymes in the *hph-1* mouse. Western blots revealed decreased amounts of TH protein in the *hph-1* brain, although levels of TRH were not documented (Hyland *et al*, 1996). In contrast, TH mRNA is reported to be elevated in substantia nigra pars compacta (SNc) tissue in the *hph-1* mouse (Zeng *et al*, 2004). However, this is may be explained as a feedback response to decreased TH protein.

Data showing attenuated TH protein in the *hph-1* mouse are supported by studies using 6-pyruvoyltetrahydropterin deficient (Pts(-/-)) (Sumi-Ichinose *et al*, 2001, 2005) and sepiapterin reductase deficient mice (SR(-/-)) (Yang *et al*, 2006). Both of these mutant mice are deficient in BH_4, and have lower levels of DA, NA and 5-HT than their wild type counterparts. TH and TRH activities were attenuated in these animals, and in Pts(-/-) mice levels of TH protein were significantly lower, although TRH concentrations were described as normal (Sumi-Ichinose *et al*, 2001). In DRD patients, decreased TH protein is also observed (Rajput *et al*, 1994; Furukawa *et al*, 1999), suggesting that diminished availability of BH_4 leads to lower amounts of TH protein, and results in decreased DA synthesis.

Sumi-Ichinose *et al* (2001) suggest that attenuated TH protein levels may arise from the action of BH_4 stabilising TH protein. They demonstrate decreased TH protein in nerve terminals of Pts(-/-) mice, but report normal levels of the protein in the cell bodies. They propose that TH forms a stable complex with BH_4 and catecholamines, and that only this stable complex is transported to the nerve terminal through axonal flow. Therefore, lower concentrations of BH_4 would mean that TH is not transported efficiently to the nerve terminal, and remains in the cell bodies of Pts(-/-) neurones.

It is unlikely that the decreased turnover of DA observed in BH_4 deficiencies results from a loss of dopaminergic terminals through degeneration. Studies of the density of striatal DA terminal sites, judged by [^{3}H]mazindol binding, showed that these sites are not altered in *hph-1* mice (Zeng *et al*, 2004). In addition, striatal aromatic L-amino acid decarboxylase (AADC) activity (Hyland *et al*, 1996) is also normal in the *hph-1* brain. Furthermore, dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2) binding is not affected in DRD (Furukawa *et al*, 1999; Huang *et al*, 2002). However, as one might expect if DA levels fall, there was a significant increase in DA D2 receptor binding in the striatum of *hph-1* mice (Zeng *et al*, 2004), which is also observed in patients with DRD (Kishore *et al*, 1998; Rinne *et al*, 2004). This suggests that feedback mechanisms exist to optimise dopaminergic neurotransmission when DA levels are attenuated in BH_4 deficiencies.

In addition to the low levels of enzyme protein accounting for differences in DA, NA and 5-HT between wild type and *hph-1* mice, some other factors may impact on the metabolism of these neurotransmitters. It has been shown that high plasma concentrations of phenylalanine can inhibit tyrosine and tryptophan crossing the blood-brain barrier (Tourian and Sidbury, 1983; Hommes and Lee, 1990), and can also competitively inhibit TH and TRH (Fernstrom and Wurtman, 1972; Medical Research Council Working Party, 1993). However, although *hph-1* mice start life with a hyperphenylalaninaemic state, their plasma levels are normalised after the first 3 weeks of its life (Bode *et al*, 1988; McDonald and Bode, 1988). Furthermore, once the phenylalanine is removed, its effects upon DA and 5-HT metabolism are unlikely to persist (Lykkelund *et al*, 1988) Therefore it is doubtful whether phenylalanine may influence *hph-1* brain monoamine levels.

In an attempt to increase monoamine production in the brains of mice and humans with BH_4 deficiencies, some studies have administrated BH_4 to patients and animals, which have met with limited success so far. Dosing *hph-1* mice subcutaneously (s.c.) every 4 hours for 24 hours with 50mg/kg BH_4 (equivalent to approximately 150μmol/kg) leads to augmentation of BH_4 in the brain, but has no effect on monoamine neurotransmitters (Hyland *et al*, 1996). In a separate study, despite elevation of BH_4 for 24 hours to values comparable to wild type with 300μmol/kg s.c. (approximately 100mg/kg), BH_4 was unable to increase monoamine turnover (Brand *et al*, 1996). It was only when the dose of BH_4 was raised to 1000μmol/kg (approximately 330mg/kg) that a stimulation of monoamine turnover was observed, when an increase in L-Dopa and 5-HTP content was detected (Brand *et al*, 1996). However, this dose proved to be fatal for a proportion (17%) of the mice, so it is unlikely that this dose would ever be used for BH_4 deficiencies in humans. *Ex vivo* analysis of TH and TRH activities revealed a modest elevation in activity for approximately 1 hour for both of these enzymes, although levels of DA and 5-HT metabolites remained raised (Brand *et al*, 1996). This suggests that a brief elevation of BH_4 can result in prolonged increases in monoamine metabolism. BH_4 has been shown to directly stimulate release of monoamine neurotransmitters from nerve terminals (Koshimura *et al*, 1994), so it is possible this could account for some of the effects seen, and Brand *et al* (1996) proffer that the prolonged increase may also result from efficient packaging of vesicles.

When Pts(-/-) mice were treated with an acute dose of intraperitoneal 50mg/kg (150 μmol/kg) BH_4, levels of 5-HT increased but DA content remained low (Sumi-Ichinose *et al*, 2001). It is suggested that this is due to differential regulation of the metabolism of DA and 5-HT. These data along with decreased TH, but not TRH protein in Pts(-/-)(Sumi-Ichinose *et al*, 2001), *hph-1* mice (Hyland *et al*, 1996), and also DRD patients (Rajput *et al*, 1994; Furukawa *et al*, 1999), provides evidence that deficient levels of BH_4 affect DA metabolism via reduced TH protein and activity, whilst BH_4 appears to directly affect 5-HT production via TRH kinetics rather than increasing expression. When Pts(-/-) mice were continuously injected with 50mg/kg BH_4 for 7 days, a significant increase in TH protein was observed, even after just 1 hour after treatment, demonstrating that the attenuation of TH protein is reversible with BH_4, but that long-term dosing may be necessary to influence DA generation.

Treatment of patients with BH_4 deficiencies with cofactor alone has only been successful in a few patients so far (Endres,1985; Blau and Thöny 1998, BH_4 deficiencies database http://www.bh4.org/BH4DatabasesBiodef.asp). Large doses are necessary (5-20mg/kg/day) because BH_4 does not readily cross the blood-brain barrier (Hoshiga *et al*, 1993). BH_4 is also relatively expensive at approximately £115 per gramme (Shircks laboratories, Jona, Switzerland). Thus, treating a 20kg child with 10mg/kg costs in the region of £8,500 per year, and will become more expensive each year. Therefore, attempting to cure the primary defect in BH_4 deficiencies by gene therapy could be very advantageous not only in terms of costs, but also if the vector was introduced into the brain, would ensure more BH_4 was present to increase the activity and expression of the NOS, TH and TRH enzymes.

3.6. CONCLUSIONS

This chapter has further demonstrated impaired BH_4 metabolism in the brain of the *hph-1* mouse. Concentrations of BH_4 in the brain are approximately 30% of wild type, and the ability of the *hph-1* biosynthetic pathway to produce BH_4 from the initial substrate GTP, is also impaired. However, biosynthetic capacity activity remains at 40% of wild type, suggesting that *hph-1* mice only have a partial deficiency of BH_4 in the brain. Activity of the BH_4 recycling pathway remains unaffected in the *hph-1* brain. This suggests that if gene transfer were used to increase GTP-CH expression and activity in *hph-1* mice, an intact recycling system exists to maintain BH_4 levels. In addition, when "DHNTP" was proposed to be introduced downstream of the *hph-1* metabolic block into the pathway, there were no differences in biosynthetic capacity between the mice, suggesting that the enzymes post-GTP-CH in the BH_4 biosynthesis pathway function normally. The lack of BH_4 also appears to impact on the concentrations of monoamine neurotransmitters. Levels of NA, DA and its metabolite HVA, as well as 5-HIAA, the metabolite of 5-HT, were all found to be lower in the *hph-1* brain. Gene transfer targeted to the *hph-1* mouse may help to increase BH_4 chronically in these animals. This may lead to increases in the level of monoamine neurotransmitters, as well as potentially correct the documented impairment of NO metabolism in the *hph-1* mouse brain. This in turn may be useful for sufferers of BH_4 deficiencies.

Chapter 4

Further biochemical characterisation of the *hph-1* mouse astrocytes

4.1. INTRODUCTION

Astrocytes are among the most common cells in the central nervous system (CNS), outnumbering neurones ten to one in most brain regions (Tsacopoulos and Magistretti, 1996), yet for decades their precise role has remained enigmatic. They were initially considered to be "brain glue", functioning as the inert cellular structure that held neurones in place and assisted their interactions (Volterra and Meldolesi, 2005). As the decades passed it was discovered they play roles in providing metabolic support for neurones (Aschner, 2000), bringing nutrients from blood vessels to cells, as well as an immune function in the CNS (Fields, 2004). More recently it was demonstrated that astrocytes synchronise neuronal firing by acting on post-synaptic receptors (Fellin *et al*, 2004), as well as dynamically controlling brain microcirculation (Zonta *et al*, 2003) and modulating synaptic transmission (Haydon, 2001).

NO derived from astrocytes may have a role in many of the functions that astrocytes and neurones perform. Astrocytes are thought to be the main source of NO in the brain, as they contain the highest concentration of L-arginine, the substrate for NOS (Aoki *et al*, 1991). Astrocytic NO can modulate synaptic plasticity via glutamatergic receptors (Kiss and Vizi, 2001; Ikeda and Murase, 2004), and may be involved in long-term potentiation (LTP) and depression (LTD), which are proposed mechanisms for learning and memory (Garthwaite and Boulton, 1995). Inducible nitric oxide synthase (iNOS) expression and NO generation in astrocytes are activated several fold by cytokines and LPS, both *in vitro* and *in vivo* (Werner *et al*, 1993; Bolaños *et al*, 1994; Bune *et al*, 1996). However, induction of iNOS and NO generation by LPS and the cytokine IFN-γ may also be toxic to astrocytes, as NO is produced in high concentrations that can inhibit the mitochondrial electron transport chain (Bolaños *et al*, 1994). Furthermore, NO produced from iNOS can diffuse to neighbouring neurones, where it can also exert toxic effects (Chao *et al*, 1996; Stewart *et al*, 2000).

NO reacts very favourably with superoxide ($\cdot O_2^-$), which leads to peroxynitrite ($ONOO^-$) formation (Beckman *et al*, 1990; Lipton *et al*, 1993). Neurones appear to be particularly susceptible to NO/$ONOO^-$ exposure (Bolaños *et al*, 1995, 1996). One

potential factor in this vulnerability may be the antioxidant capacity of these cell types. The intracellular concentration of reduced glutathione (GSH) appears to be an important aspect in dictating the susceptibility of cells to $NO/ONOO^-$ exposure (Bolaños *et al*, 1996). GSH can potentially nullify the deleterious effects of $NO/ONOO^-$ (Quijano *et al*, 1997; Hughes, 1999). Astrocytes have approximately double the intracellular concentration of GSH found in neurones (Bolaños *et al*, 1995), which may be accounted for by the 9-fold greater specific activity of glutamate cysteine ligase, the rate limiting step in GSH production, in astrocytes compared to neurones (Gegg *et al*, 2003). It is thought that astrocytes may protect neurones by indirectly trafficking GSH to neurones via the multi-drug resistance protein (MRP1)(Hirrlinger *et al*, 2002). When neurones are cultured alone, intracellular GSH concentration is significantly lower than when co-cultured with astrocytes (Bolaños *et al*, 1996; Dringen *et al*, 1999), and when astrocytes are exposed to NO, the intracellular GSH concentration and release increases (Gegg *et al*, 2003). Thus, astrocytes would appear to have an important role in protecting neurones from oxidative stress, but large quantities of NO produced from these cells may also be deleterious.

In order to increase NO production in astrocytes following induction of iNOS expression, adequate amounts of the cofactor BH_4 are required (Sakai *et al*, 1995). LPS + IFN-γ stimulation of iNOS expression is accompanied by a parallel induction of GTP-CH expression in cultured cells (Hattori and Gross, 1993; Bune *et al*, 1996), and inhibition of BH_4 synthesis is reported to decrease NO (Gross and Levi, 1992; Schoedon *et al*, 1994). In *hph-1* astrocytes, impaired BH_4 metabolism results in decreased NO generation when these cells are stimulated with LPS + IFN-γ (Barker *et al*, 1998), even though protein levels of iNOS are reported to be significantly higher than wild type (Barker *et al*, 1998). At low concentrations of BH_4, purified NOS protein has been reported to produce $\cdot O_2^-$ and hydrogen peroxide (H_2O_2)(Heinzel *et al*, 1992, Rosen *et al*, 2002), therefore it is possible that in *hph-1* astrocytes NO and $\cdot O_2^-$ are generated, which can combine together to form the toxic species $ONOO^-$ (Beckman *et al*, 1990). Indeed, *hph-1* mice have decreased concentrations of GSH in the brain and kidney, which may be attributed to a greater consumption of GSH in *hph-1* compared to WT by reactive oxygen and nitrogen species (Lam, 2004).

In patients with inborn errors of BH_4 metabolism, decreased concentrations NO_2^- and NO_3^- have been reported in cerebrospinal fluid (CSF)(Heales *et al*, 1999; Zorzi *et al*, 2002a), which suggests an impaired formation of NO in these patients. The *hph-1* mouse is reported to be a good model for inborn errors of BH_4 metabolism, in particular dominantly inherited GTP-CH deficiency dopa responsive dystonia (Hyland *et al*, 2003). Therefore studying *hph-1* mouse astrocytes further may yield vital information about the effect of impaired BH_4 and NO metabolism in the pathogenesis of these diseases, and be useful as a cellular model for gene transfer in BH_4-deficient states.

4.2. AIMS

In view of the fact that *hph-1* astrocytes have previously been demonstrated to have impaired BH_4 metabolism, and show an attenuated response to stimuli that increase GTP-CH and iNOS expression, the aims of this chapter are as follows:

- Further characterise impaired BH_4 and NO metabolism in *hph-1* astrocytes, by measuring BH_4 content and NO production (as judged by NO_2^- and NO_3^- release into the extracellular media) under basal, and LPS + IFN-γ stimulated conditions.
- Investigate the ability of *hph-1* astrocytes to produce BH_4 from L-sepiapterin that can be converted to BH_4 by the salvage pathway.
- Investigate whether increasing BH_4 in *hph-1* astrocytes can augment their response to LPS + IFN-γ stimulated NO production.

4.3. METHODS

4.3.1. Cell culture

Primary astrocytes were cultured from wild type and *hph-1* mouse neonates as described in section 2.2.2. They were treated *in vitro* on day 13 as described in section 2.2.4.

4.3.2. Tetrahydrobiopterin measurement

Cells were prepared for analysis of BH_4 and measured as described in section 2.3.4 and section 2.1 respectively.

4.3.3. Nitrite and nitrate measurement

NO_2^- and NO_3^- were measured in the cell culture media of wild type and *hph-1* astrocytes as an index of NO production, which is described in section 2.6.

4.3.4. Protein concentration

Protein concentration in cells used for BH_4 and NO_2^- and NO_3^- measurements was calculated based on the method of Lowry *et al* (1951) described in section 2.9, using a standard curve of BSA dissolved in 0.5 M NaOH, with concentrations of 0.05 mg/ml to 0.6 mg/ml..

4.3.5. Experimental protocol

For experiments involving LPS and/or IFN-γ stimulation, cells were either stimulated with IFN-γ (100 U/ml) or LPS (1μg/ml), or a combination of the two, with vehicle media as a control. In addition to LPS + IFN-γ alone, this treatment was combined with L-NIL (N6-iminoethyl-L-Lysine) (100μM) a specific iNOS inhibitor (Wolff *et al*, 1998), or L-sepiapterin (1μM), a substrate for the BH_4 salvage pathway enzymes. In other studies, astrocytes received increasing concentrations of L-sepiapterin (0.2μM – 2μM), or vehicle media as a control. Cells were harvested 24 hours after treatment.

4.3.6. Statistical analysis

Statistical tests were conducted as described in section 2.10.

4.4. RESULTS

4.4.1. Comparison of wild type and *hph-1* mice astrocytes under basal conditions

Table 4.1 shows that BH_4 in untreated *hph-1* astrocytes is significantly lower than in wild type astrocytes ($p < 0.01$, $n = 9$). BH_4 levels in the *hph-1* cells (3.61 ± 0.45pmol/ mg protein) were 47% of wild type (7.57 ± 1.08pmol/mg protein).

By contrast table 4.1 shows that NO_2^- and NO_3^- in untreated *hph-1* astrocyte cell culture media was not significantly lower than wild type ($p = 0.34$, $n = 8$).

4.4.2. Effect of Lipopolysaccharide and Interferon-γ on tetrahydrobiopterin, and nitrite and nitrate levels in wild type and *hph-1* astrocytes

Wild type and *hph-1* astrocytes were treated with combinations of the bacterial endotoxin LPS and cytokine IFN-γ, to compare the effects of stimuli that increase GTP-CH and iNOS expression, on BH_4 and NO_2^- and NO_3^- levels. IFN-γ had no effect on basal BH_4 levels in either wild type or *hph-1* astrocytes (figure 4.1). LPS induced an approximately 19-fold increase in wild type BH_4 (146.92 ± 16.34pmol/mg protein) and a 3-fold increase in *hph-1* cells (10.72 ± 1.15pmol/mg protein). These increases were statistically significant ($p < 0.001$, $n = 7$-9 for wild type and $p < 0.001$, $n = 9$-12 for *hph-1*). The combination of LPS + IFN-γ also significantly increased BH_4 in wild type (156.88 ± 24.63pmol/mg protein, $p < 0.001$, $n = 9$-12) and *hph-1* astrocytes (11.41 ± 0.96pmol/mg protein, $p < 0.001$, $n = 9$-12), however this combination did not significantly raise BH_4 above LPS stimulated levels for either wild type or *hph-1* astrocytes.

These data were compared to see if *hph-1* LPS + IFN-γ-stimulated BH_4 levels were different to wild type. *hph-1* astrocytes treated with LPS and LPS + IFN-γ produced significantly lower levels of BH_4 than wild type ($p < 0.001$, $n = 7$-12). The increase in *hph-1* astrocyte BH_4 was approximately 7% of the total BH_4 produced by wild type cells for both treatments.

LPS + IFN-γ also stimulates the expression of iNOS, which generates NO that can be indirectly measured by assaying the levels of NO_2^- and NO_3^-, metabolites of NO, in

	wild type	*hph-1*
BH_4 pmol/mg protein	7.57 ± 1.08	3.61 ± 0.45*
NOx nmol/mg protein	2.43 ± 0.66	1.99 ± 0.75

Table 4.1. Basal levels of BH_4 and NO_2^- + NO_3^- in wild type and *hph-1* cortical astrocytes.

BH_4 was measured using reverse-phase HPLC coupled with ECD. NO_2^- + NO_3^- concentrations were measured using the Griess reaction. Data are expressed as mean ± SEM of 8-9 independent cell culture preparations. * = $p < 0.01$ vs. wild type, calculated using Student's t-test.

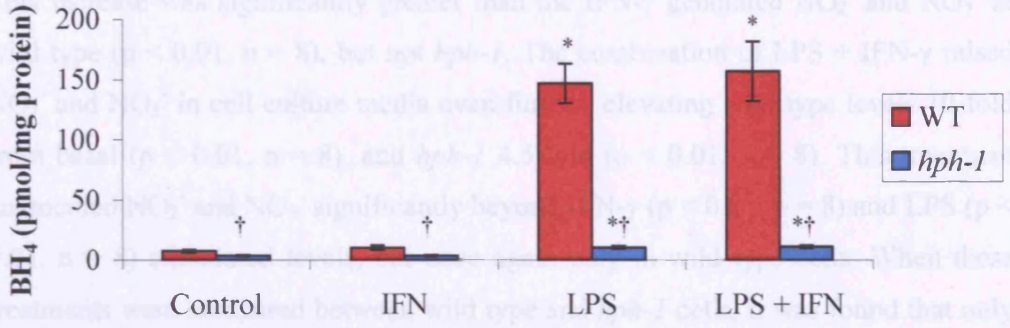


Figure 4.1. Effect of combinations of LPS (100 U/ml), IFN-γ (IFN, 1μg/ml), and LPS + IFN-γ (LPS + IFN) on BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

Cells were treated for 24 hours. BH_4 was measured using reverse-phase HPLC coupled with ECD. Data are expressed as mean ± SEM of 7-12 independent cell culture preparations. * = $p < 0.001$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.001$ vs. wild -type calculated using Student's t-test.

cell culture media. When cells were treated with IFN-γ, an approximate 2-fold increase in NO_2^- and NO_3^- was seen in wild type cell culture media ($p < 0.05$, $n = 8$), with a 3-fold rise observed in *hph-1* ($p < 0.01$, $n = 4$-8). LPS induced significant increases in NO_2^- and NO_3^- in both wild type ($p < 0.01$, $n = 8$) and *hph-1* astrocytes ($p < 0.01$, $n = 4$-8), to approximately 5-fold and 3-fold of basal levels, respectively. This increase was significantly greater than the IFN-γ generated NO_2^- and NO_3^- in wild type ($p < 0.01$, $n = 8$), but not *hph-1*. The combination of LPS + IFN-γ raised NO_2^- and NO_3^- in cell culture media even further, elevating wild type levels 10-fold from basal ($p < 0.01$, $n = 8$), and *hph-1* 4.5-fold ($p < 0.01$, $n = 8$). This treatment augmented NO_2^- and NO_3^- significantly beyond IFN-γ ($p < 0.01$, $n = 8$) and LPS ($p < 0.01$, $n = 8$) stimulated levels, but once again only in wild type cells. When these treatments were compared between wild type and *hph-1* cells, it was found that only with the combination of LPS + IFN-γ was NO_2^- and NO_3^- in *hph-1* astrocytes significantly different from wild type ($p < 0.05$, $n = 8$), at around 36% lower (figure 4.2).

4.4.3. Effect of N6-iminoethyl-L-Lysine on Lipopolysaccharide and Interferon-γ induced increases in *hph-1* and wild type astrocyte tetrahydrobiopterin and nitrite and nitrate levels

The iNOS inhibitor L-NIL was added to wild type and *hph-1* astrocytes, to observe whether the increases in BH_4 or NO_2^- and NO_3^- following LPS plus IFN-γ stimulation were dependent on iNOS activity (Wolff *et al*, 1998). L-NIL did not significantly affect LPS + IFN-γ stimulated BH_4 increases in wild type (83.64 ± 31.61 pmol/mg protein vs. 90.72 ± 32.59 pmol/mg protein for L-NIL treated, $n = 7$) (figure 4.3) or *hph-1* astrocytes (4.25 ± 1.61 pmol/mg protein vs. 4.12 ± 1.56 pmol/mg protein for L-NIL treated, $n = 7$). However, the increases in NO_2^- and NO_3^- following LPS + IFN-γ treatment were significantly attenuated by L-NIL in wild type ($p < 0.01$, $n = 8$) and *hph-1* astrocytes ($p < 0.01$, $n = 7$-8), so that they no longer differed from basal levels (figure 4.4).

4.4.4. Effect of L-sepiapterin on tetrahydrobiopterin and nitrite and nitrate levels in wild type and *hph-1* astrocytes

Wild type and *hph-1* astrocytes were treated with increasing concentrations of L-sepiapterin, from 0.2-2.0μM, to investigate the ability of the *hph-1* cells to produce

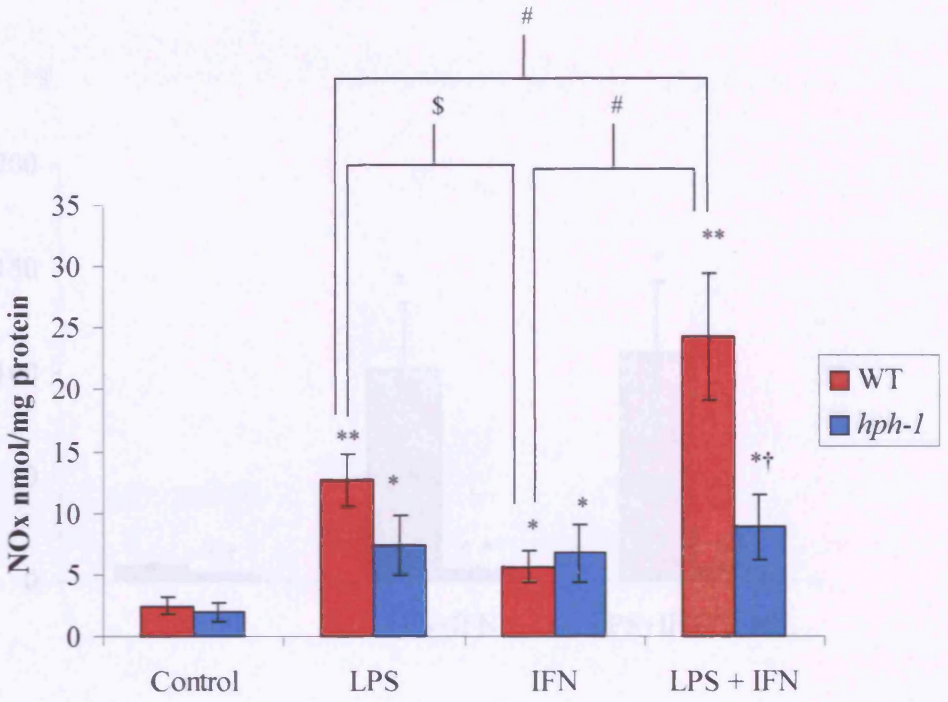


Figure 4.2. Effect of combinations of LPS (100 U/ml) + IFN-γ (IFN, 1μg/ml) on NO_2^- + NO_3^- (NOx) production in cell culture media of wild type (WT) and *hph-1* cortical astrocytes. Cells were treated for 24 hours and NOx production was measured using the Griess assay (see section 2.6).

Data are expressed as mean ± SEM of 4-8 independent cell culture preparations. * = $p < 0.05$ vs. untreated control, ** = $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. $^{\$}$ = $p < 0.01$ vs. LPS stimulated calculated using one way ANOVA followed by least significant difference test. $^{\#}$ = $p < 0.01$ vs. LPS + IFN-γ stimulated calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild type calculated using Student's t-test.

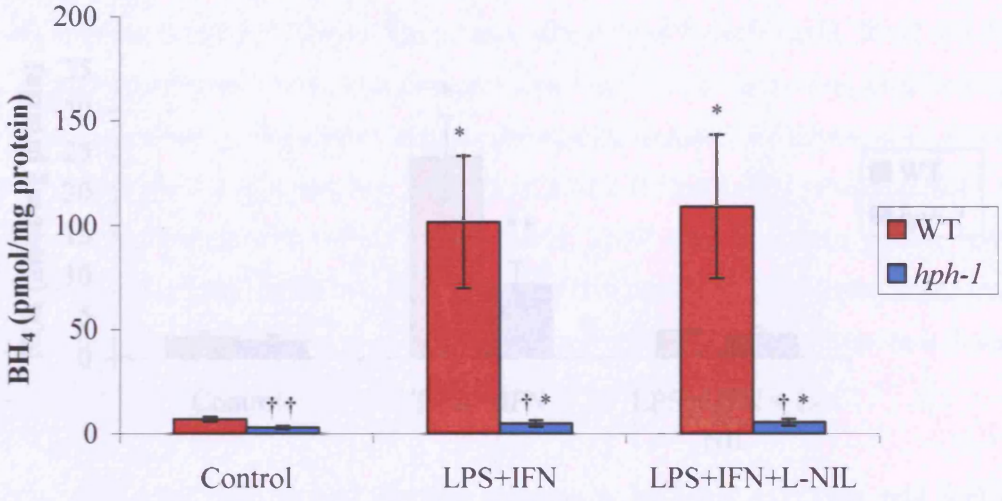


Figure 4.3. Effect of LPS (100 U/ml) + IFN-γ (IFN, 1μg/ml), and LPS + IFN-γ + N6-iminoethyl-L-lysine (L-NIL, 100μM) on BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

Cells were treated for 24 hours. BH_4 was measured using reverse-phase HPLC coupled with ECD. Data are expressed as mean ± SEM of 6-10 independent cell culture preparations. † = $p < 0.05$ vs. wild-type, † † = $p < 0.01$ vs. wild-type calculated using Student's t-test. * = $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type, † † = $p < 0.01$ vs. wild-type calculated using Student's t-test.

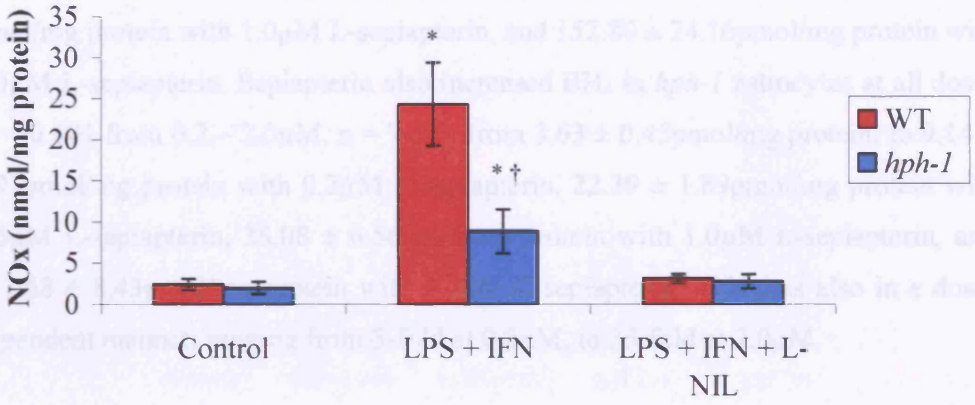


Figure 4.4. Effect of LPS (100 U/ml) + IFN-γ (IFN, 1μg/ml) stimulation, and inhibition of LPS + IFN stimulation with N6-iminoethyl-L-lysine (L-NIL, 100μM), on NO_2^- + NO_3^- (NOx) production in cell culture media of wild type (WT) and *hph-1* cortical astrocytes. Cells were treated for 24 hours. LNIL was administered at a concentration of 100μM.

NO_2^- + NO_3^- concentrations were measured using the Griess reaction. Data are expressed as mean ± SEM of 5-8 independent cell culture preparations. * = $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type calculated using Student's t-test.

BH_4 via other pathways than the *de novo* biosynthesis (i.e. the salvage pathway)(figure 4.5). In wild type astrocytes all doses of L-sepiapterin increased BH_4 ($p < 0.01$ for 0.2, 0.5 and 2.0μM, $p < 0.001$ for 1.0μM, n = 7-12) in a dose-related manner, from approximately a 2-fold increase at 0.2μM to 20-fold for 2.0μM. BH_4 rose from 7.57 ± 1.08pmol/mg protein, to 16.23 ± 1.69pmol/mg protein with 0.2μM L-sepiapterin, 33.31 ± 3.22pmol/mg protein with 0.5μM L-sepiapterin, 59.42 ± 8.35 pmol/mg protein with 1.0μM L-sepiapterin, and 152.80 ± 24.16pmol/mg protein with 2.0μM L-sepiapterin. Sepiapterin also increased BH_4 in *hph-1* astrocytes at all doses ($p < 0.001$ from 0.2 – 2.0μM, n = 7-12), from 3.63 ± 0.45pmol/mg protein, to 9.14 ± 0.91pmol/mg protein with 0.2μM L-sepiapterin, 22.39 ± 1.89pmol/mg protein with 0.5μM L-sepiapterin, 38.08 ± 6.56pmol/mg protein with 1.0μM L-sepiapterin, and 117.68 ± 8.43pmol/mg protein with 2.0μM L-sepiapterin. This was also in a dose-dependent manner, ranging from 3-fold at 0.2μM, to 33-fold at 2.0μM.

These data were also studied for any differences between wild type and *hph-1* astrocytes in their response to L-sepiapterin, and at all doses tested except the highest (2.0μM), the increase in BH_4 shown by *hph-1* astrocytes was significantly lower than the wild type ($p < 0.05$, n = 7-12).

Levels of NO_2^- and NO_3^- were also monitored following a dose of 1μM sepiapterin. No significant effects were seen in wild type or *hph-1* astrocytes with this treatment (figure 4.7).

4.4.5. Effect of the combination of Lipopolysaccharide and Interferon-γ with L-sepiapterin on *hph-1* and wild type astrocyte tetrahydrobiopterin and nitrite and nitrate levels

L-sepiapterin was given in combination with LPS and IFN-γ to investigate whether stimuli that increase BH_4 via different pathways could summate to give larger increases in BH_4, and to investigate whether this would lead to increased NO_2^- and NO_3^- in *hph-1* cells. Figure 4.6 shows the BH_4 response of *hph-1* and wild type astrocytes to these stimuli. In wild type and *hph-1* astrocytes both L-sepiapterin and LPS + IFN-γ increase BH_4 to varying extents, as described above in section 4.3.2. The combination of LPS + IFN-γ + Sepiapterin in wild type cells increased BH_4 above control ($p < 0.05$, n = 8-10) and sepiapterin ($p < 0.05$, n = 7-8) stimulated

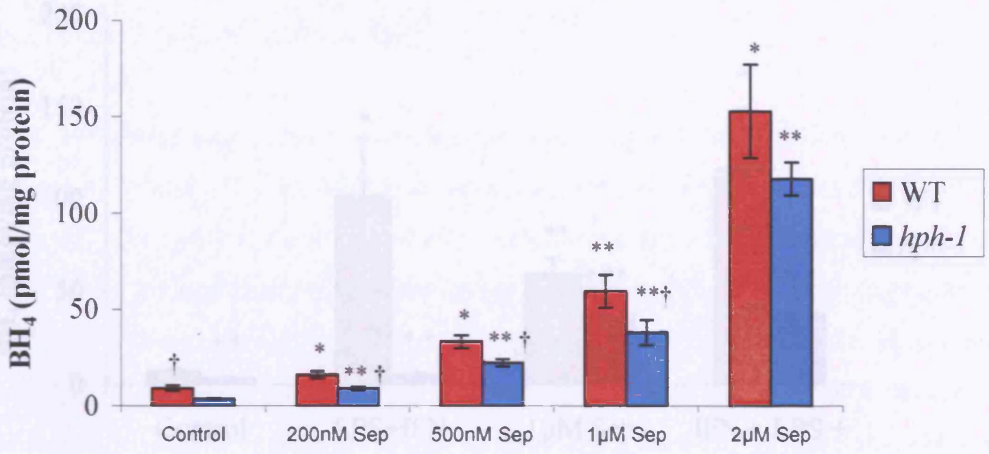


Figure 4.5. Effect of increasing concentration of L-Sepiapterin (Sep) on BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

Cells were treated for 24 hours. BH_4 was measured using reverse-phase HPLC coupled with ECD. Data are expressed as mean ± SEM of 7-12 independent cell culture preparations. * = $p < 0.01$ vs. untreated control, ** = $p < 0.001$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type calculated using Student's t-test.

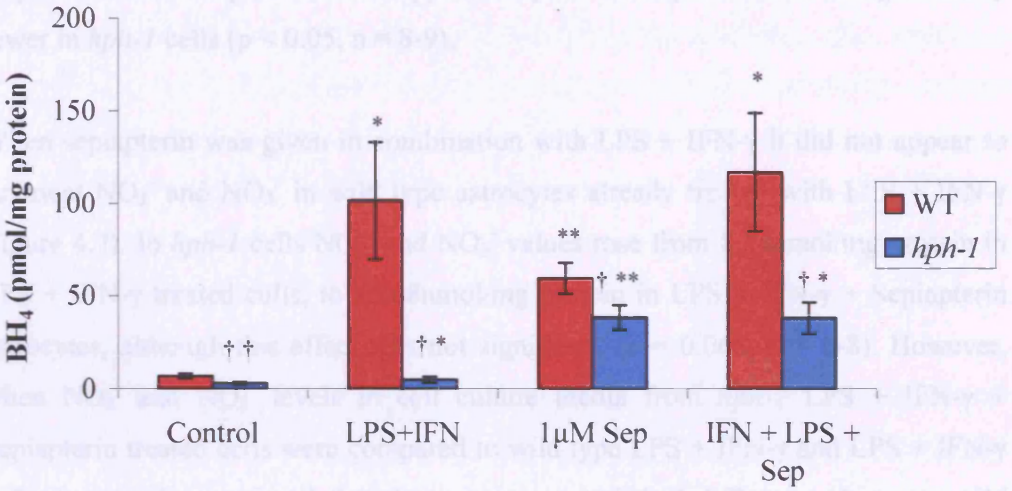


Figure 4.6. Effect of LPS (100 U/ml) + IFN-γ (IFN, 1μg/ml), and L-sepiapterin (Sep, 1μM) on BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

Cells were treated for 24 hours. BH_4 was measured using reverse-phase HPLC coupled with ECD. Data are expressed as mean ± SEM of 6-10 independent cell culture preparations. * = $p < 0.01$ vs. untreated control, ** = $p < 0.001$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type, † † = $p < 0.01$ vs. wild-type calculated using Student's t-test.

levels, but did not exceed LPS + IFN-γ values. In contrast, in *hph-1* astrocytes the combination of LPS + IFN-γ + Sepiapterin significantly increased BH_4 above control ($p < 0.01$, n = 6-9) and LPS + IFN-γ stimulated ($p < 0.01$, n = 9-10), but did not increase BH_4 above sepiapterin stimulated levels. When LPS + IFN-γ + Sepiapterin responses were compared in wild type and *hph-1* astrocytes, BH_4 was significantly lower in *hph-1* cells ($p < 0.05$, n = 8-9).

When sepiapterin was given in combination with LPS + IFN-γ it did not appear to augment NO_2^- and NO_3^- in wild type astrocytes already treated with LPS + IFN-γ (figure 4.7). In *hph-1* cells NO_2^- and NO_3^- values rose from 8.84nmol/mg protein in LPS + IFN-γ treated cells, to 15.68nmol/mg protein in LPS + IFN-γ + Sepiapterin astrocytes, although this effect was not significant ($p = 0.068$, n = 6-8). However, when NO_2^- and NO_3^- levels in cell culture media from *hph-1* LPS + IFN-γ + Sepiapterin treated cells were compared to wild type LPS + IFN-γ and LPS + IFN-γ + Sepiapterin it was found that there were no statistical differences between wild type and *hph-1* groups (figure 4.7).

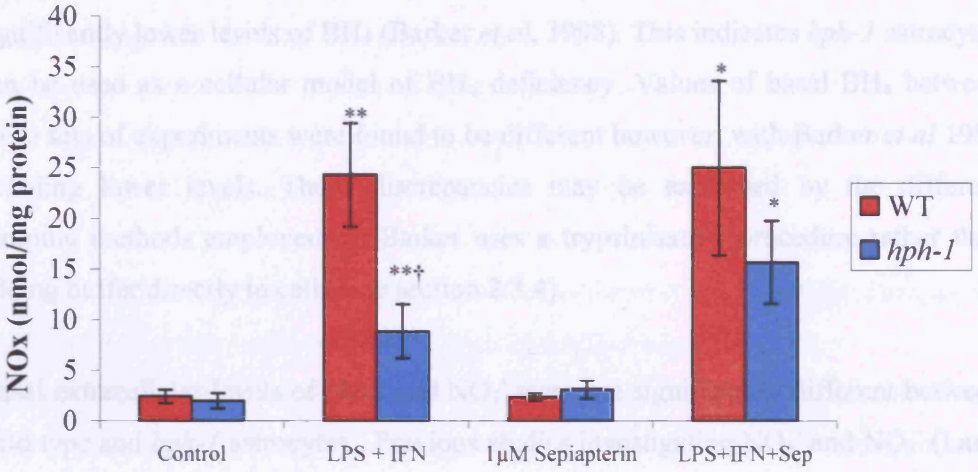


Figure 4.7. Effect of combinations of LPS (100 U/ml) + IFN-γ (IFN, 1μg/ml) and sepiapterin (1μM) on NO_2^- + NO_3^- (NOx) production in cell culture media of wild type (WT) and *hph-1* cortical astrocytes. Cells were treated for 24 hours. NO_2^- + NO_3^- concentrations were measured using the Griess reaction.

Data are expressed as mean ± SEM of 5-8 independent cell culture preparations. * = $p < 0.05$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. ** = $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type calculated using Student's t-test.

4.5. DISCUSSION

4.5.1. Comparison of wild type and *hph-1* mice astrocytes under basal conditions

In agreement with existing literature, *hph-1* astrocytes were found to have significantly lower levels of BH_4 (Barker *et al*, 1998). This indicates *hph-1* astrocytes can be used as a cellular model of BH_4 deficiency. Values of basal BH_4 between these sets of experiments were found to be different however, with Barker *et al* 1998 detailing lower levels. These discrepancies may be explained by the different scraping methods employed, as Barker uses a trypsinisation procedure rather than adding buffer directly to cells (see section 2.3.4).

Basal extracellular levels of NO_2^- and NO_3^- were not significantly different between wild type and *hph-1* astrocytes. Previous studies investigating NO_2^- and NO_3^- (Lam, 2004) and also direct measurements of NO under basal conditions (Barker *et al*, 1998) were unable to detect NO_2^- and NO_3^- or NO in wild type or *hph-1* astrocytes. Basal NO_2^- and NO_3^- levels in this study were at the limit of detection of the assay, and given that the Griess assay does not discriminate over the source of NO_2^- or NO_3^- (Green *et al*, 1982), it is possible that combinations of these factors is why equal NO_2^- and NO_3^- are detected in wild type and *hph-1* cell culture media. Therefore, further work using a highly sensitive direct assay of NO may show discrepancies in NO metabolism between *hph-1* and wild type under basal conditions. Additionally, measurement of the formation of cGMP, a downstream target of NO (Ignarro *et al*, 1981), could reveal differences in NO metabolism, as cGMP levels are influenced by the concentration of BH_4 in the cell (Muhl *et al*, 1994; Delgado-Esteban *et al*, 2002), and have been measured under basal conditions in mouse astrocytes (Yeung *et al*, 1992, 1996).

Alternatively, it may be that BH_4 is not limiting for NOS in *hph-1* astrocytes under basal conditions. The K_m of BH_4 for NOS is relatively low at typically 0.02μM (Giovanelli *et al*, 1991), in contrast to 2μM for PAH for example (Abita *et al*, 1984). Therefore there may be sufficient BH_4 to allow NOS isoforms to function normally in these cells. However, it is not possible to ascertain this from the BH_4 concentration measured, and a study of the K_m of BH_4 for nNOS in *hph-1* cells would need to be

done to investigate this. Astrocytes are thought to be the main source of NO in the brain, as they contain the highest concentration of L-arginine, the substrate for NOS (Aoki *et al*, 1991). Previous studies show that nNOS specific activity is decreased in *hph-1* brains in the absence of exogenous cofactor (Brand *et al*, 1995), and in patients with inborn errors of BH_4 metabolism, decreased concentrations of NO_2^- and NO_3^- are reported in CSF (Heales *et al*, 1999; Zorzi *et al*, 2002a). Therefore, although there were no differences detected between basal levels of NO_2^- and NO_3^- in *hph-1* and wild type astrocytes, attenuated production of NO in *hph-1* brains must arise from somewhere, and astrocytes are potential sources of this.

4.5.2. Effect of Lipopolysaccharide and Interferon-γ on tetrahydrobiopterin, and nitrite and nitrate levels in wild type and *hph-1* astrocytes

Various effects were observed following exposure of wild type and *hph-1* astrocytes to combinations of LPS and the cytokine IFN-γ. IFN-γ alone had no effect on basal BH_4 in wild type or *hph-1* astrocytes, whilst LPS induced a 19-fold increase in wild type and a 3-fold increase in *hph-1* BH_4. The combination of the inflammatory mediators also increased BH_4 in both types of astrocytes, but not above LPS stimulated levels. This suggests increases in BH_4 derive from LPS stimulation, not IFN-γ. These results agree with studies in rat astrocytes, where IFN-γ has no effect on BH_4 concentrations, and the combination of LPS + IFN-γ does not significantly increase BH_4 values above LPS-stimulated (Sakai *et al*, 1995). The inability of IFN-γ to increase BH_4 may be because it does not down-regulate GTP-CH feedback regulatory protein (GFRP) levels (Werner *et al*, 2002). In human THP-1 cells and in rats *in vivo*, LPS attenuates the expression of GFRP, an inhibitory complex that binds and regulates GTP-CH. IFN-γ had no effect on GFRP in these cells (Werner *et al*, 2002).

BH_4 levels between wild type and *hph-1* astrocytes treated with LPS and LPS + IFN-γ were markedly different. In *hph-1* stimulated cells BH_4 was significantly lower than the wild type, an effect consistent with Barker *et al*, 1998 who describe doubling of BH_4 in wild type and *hph-1* astrocytes following LPS + IFN-γ, but *hph-1* levels remained 25% of wild type. The magnitude of response is different between the data presented here and by Barker *et al*, 1998, again possibly due to different methods used to harvest the cells (see section 2.3.4). LPS + IFN-γ stimulation

increases GTP-CH activity in human endothelial cells, but not the other enzymes in the biosynthetic pathway (Werner-Felmayer *et al*, 1993). Therefore, these results demonstrate further that *hph-1* mice have an impaired ability to generate BH_4 by *de novo* synthesis due to their deficiency in GTP-CH.

When NO_2^- and NO_3^- were measured in *hph-1* and wild type astrocyte cell culture media following stimulation with combinations of LPS and IFN-γ, the reported effects were comparable with published results (Bolaños *et al*, 1994). In wild type cells, IFN-γ increased NO_2^- and NO_3^- above basal levels, and LPS amplified this further, significantly exceeding IFN-γ stimulation. LPS + IFN-γ together increased NO_2^- and NO_3^- in wild type beyond LPS or IFN-γ levels, such that LPS + IFN-γ stimulation produced more NO_2^- and NO_3^- than the sum of its separate parts. This effect is consistent with data reported in rat astrocytes, where the LPS + IFN-γ had a greater effect than LPS and IFN-γ combined (Bolaños *et al*, 1994). In *hph-1* culture media, all three treatments elevated NO_2^- and NO_3^- above basal, however none of these changes were significantly different from each other. When wild type and *hph-1* were compared it was discovered that only with LPS + IFN-γ together were there any differences between the two types of astrocyte, where NO_2^- and NO_3^- production was significantly impaired in *hph-1* cells. These data agree with the results of Barker *et al*, 1998 who reported lower amounts of NO produced from *hph-1* astrocytes, confirming *hph-1* cells have an impaired ability to generate NO when stimulated with LPS + IFN-γ. From these data it was decided to use both LPS + IFN-γ in any further experiments involving iNOS stimulation.

The stimulation of iNOS expression by cytokines and bacterial agents such as LPS is well documented. A number of different cells respond to LPS + IFN-γ with increases in BH_4, NO, and cGMP (Hattori and Gross, 1993; Sakai *et al*, 1995; Ding *et al*, 1997 Pedraza *et al*, 2001, 2003), including rat (Simmons and Murphy, 1992; Bolaños *et al*, 1994; Choi *et al*, 2002) and mouse astrocytes (Barker *et al*, 1998). LPS also induces BH_4 and NO production in rats *in vivo* (Bune *et al*, 1996). Studies examining the time course of LPS + IFN-γ stimulation demonstrated that BH_4 and NO generation, as well as GTP-CH and NOS expression, are induced in parallel (Hattori and Gross, 1993). It is thought production of NO is dependent on BH_4, as DAHP prevents NO generation in cells treated with LPS + IFN-γ (Hattori and Gross, 1993), whereas the

iNOS inhibitor L-NIL has no effect on BH_4 (section 4.4.3). However, while induction of GTP-CH by LPS + IFN-γ is necessary for an increase in iNOS activity and NO generation, iNOS mRNA is still induced in the presence of DAHP, suggesting that LPS + IFN-γ induces iNOS expression independently of BH_4 production (Togari *et al*, 1998).

These data raise questions about why limiting BH_4 production in *hph-1* astrocytes leads to impaired NO_2^- and NO_3^- following LPS + IFN-γ stimulation. Work in the whole brain of *hph-1* mice shows that in the absence of exogenous BH_4, the K_m for arginine is significantly increased in *hph-1* brain, and NOS specific activity is attenuated by 20% (Brand *et al*, 1995). However this work examines nNOS the constitutive isoform of NOS in the brain, and it is not clear whether this extends to iNOS. Another explanation for lower LPS + IFN-γ stimulated NO_2^- and NO_3^- in *hph-1* cells may be due to the role of BH_4 in iNOS dimerisation. iNOS is only active as a homodimer (Ghosh *et al*, 1995), and BH_4 is required for dimerisation (Baek *et al*, 1993). BH_4 promotes the stabilisation of iNOS into the active dimer form, leading to increased NO (Tzeng *et al*, 1995). L-arginine also promotes dimerisation of iNOS (Baek *et al*, 1993), so perhaps the combination of low BH_4 leading to increased K_m of arginine for iNOS, and decreased dimerisation of iNOS due to low BH_4 and arginine binding, means that *hph-1* astrocytes produce less NO following stimulation.

Barker *et al* 1998 demonstrate that iNOS protein and activity is increased following LPS + IFN-γ stimulation in *hph-1* astrocytes. They suggest this higher activity arises from the effect NO has on limiting NFκB-dependent iNOS expression (Park *et al*, 1997). Therefore lower production of NO in *hph-1* cells leads to increased iNOS activity. However, rat hepatocytes pre-treated with an NO-donor prior to cytokine stimulation, had increased iNOS activity and NO_2^- and NO_3^- but no change in iNOS protein (Park *et al*, 2002). This effect may be cell specific, as macrophages and smooth muscle cells did not respond to the NO-donor. In the same study, pre-treatment with the NO-donor suppressed GFRP expression, giving augmented GTP-CH activity and BH_4 production, in parallel with increased NO_2^- and NO_3^- and iNOS dimerisation. So perhaps there is less suppression of GRFP in *hph-1* cells, indirectly resulting in attenuated NO production following stimulation.

A further factor to consider could be arginine uptake into cells. Expression of type 2 cationic amino acid transport proteins (CAT-2), which support cellular arginine uptake, increase with rising concentrations of BH_4 in rat cardiomyocytes (Schwartz *et al*, 2001). Treating these cells with LPS increased arginine uptake and NO_2^- and NO_3^- generation, but only in the presence of BH_4. Stevens *et al*, 1996 describe the expression of CAT-2 in rat astrocytes, and show that iNOS and CAT-2 expression, arginine uptake and nitrate production all increase concomitantly following LPS + IFN-γ stimulation. However these results do not document BH_4 levels or GTP-CH expression or activity, so one can only tentatively suggest lower BH_4 levels leads to impaired arginine transport, and thus lower iNOS activity and NO generation in *hph-1* astrocytes.

A number of factors, including the effect of BH_4 concentration on iNOS dimerisation, arginine binding and uptake, NO suppression of GFRP, and expression and activity of iNOS are likely to be involved in the results reported here. However, further work is necessary to elucidate factors governing impaired NO_2^- and NO_3^- generation following LPS + IFN-γ stimulation in *hph-1* astrocytes.

4.5.3. Effect of N6-iminoethyl-L-Lysine on Lipopolysaccharide and Interferon-γ induced increases in *hph-1* and wild type astrocyte BH_4 levels

The iNOS inhibitor L-NIL did not inhibit increases in BH_4 by LPS + IFN-γ stimulation. L-NIL is a relatively selective inhibitor of iNOS (Moore *et al*, 1994; Wolff *et al*, 1998; Alderton *et al*, 2001), suggesting that BH_4 synthesis is not modulated by NO produced by iNOS. However, the increase in NO_2^- and NO_3^- following LPS + IFN-γ stimulation was iNOS dependent, as L-NIL prevented the effect in both cell types. This suggests that the product of LPS + IFN-γ stimulation is mostly NO in this experiment, as L-NIL abolished any increases in NO_2^- and NO_3^-.

4.5.4. Effect of L-sepiapterin on tetrahydrobiopterin and nitrite and nitrate levels in wild type and *hph-1* astrocytes

L-sepiapterin increases BH_4 in wild type and *hph-1* astrocytes dose-dependently from 0.2-2μM. In addition, pilot studies using 100μM sepiapterin produced approximately 6nmol/mg protein BH_4 in wild type astrocytes (data not shown), suggesting the relationship is dose dependent even at higher concentrations.

Sepiapterin is converted to BH_4 via two enzymic steps; firstly by SR to 7,8-dihydrobiopterin, and then to BH_4 by DHFR (Nichol *et al*, 1983)(figure 1.4). Unlike GTP-CH (Harada *et al*, 1993; Milstien *et al* 1996) it has not been recorded that SR or DHFR are under the control of any regulatory protein or feedback inhibition. Thus, at the concentrations of sepiapterin used a plateau of BH_4 production is not reached.

It was observed that at concentrations up to 2μM *hph-1* cells generated significantly lower levels of BH_4 than wild type. This effect is not documented so far, and a number of explanations for this may exist. One possibility is that expression or activity of SR or DHFR are down-regulated in *hph-1* cells in response to reduced GTP-CH. Evidence for this is observed in human neuroblastoma cell lines treated with α-synuclein (Baptista *et al*, 2003). α-synuclein is linked to dopaminergic cell death in Parkinson's disease, and cells treated with this were found to have lower levels of both GTP-CH and SR mRNA and protein. In addition to decreased GTP-CH and SR activity, diminished expression of the orphan nuclear receptor Nurr1 was observed (Baptista *et al*, 2003). Nurr1 is a transcription factor involved in the co-ordinate regulation of DA biosynthesis, and regulates genes involved in DA synthesis including those for GTP-CH and SR. This could be a mechanism for a reduction in SR that accompanies decreased GTP-CH expression.

Evidence of modulation of SR with GTP-CH expression is also seen in murine neuroblastoma cells (Mori *et al*, 1997), and rat thymocytes (Schott *et al*, 1992) following LPS stimulation, where parallel increases in GTP-CH and SR expression occur. Lectin, IFN-γ and Interleukin-2 also stimulate the activity of both GTP-CH and SR (Kerler *et al*, 1989; Ziegler *et al*, 1990). However some sources report changes in GTP-CH but not SR activity. Rat phaeochromocytoma PC12 cells stimulated with epidermal growth factor (EGF), nerve growth factor (NGF) and IFN-γ, have increased GTP-CH but not SR activity (Anastasiadis *et al*, 1996). Also, patients with the autosomal dominant form of GTP-CH deficiency (DRD) have decreased activity and levels of GTP-CH, but SR activity within the normal range (Blau *et al*, 2001b). No data is available for expression levels of SR in DRD, or in the autosomal recessive form of GTP-CH deficiency to give insight into how SR may be affected by GTP-CH down-regulation. Maier *et al* (1993) suggest steady state

levels of mRNA regulate SR activity, so perhaps this is one mechanism for the observed effects.

Another explanation for the reduction in BH_4 in *hph-1* astrocytes following L-sepiapterin treatment, is that DHFR, the other enzyme involved in conversion of L-sepiapterin to BH_4, may have attenuated expression or activity. Unfortunately evidence for altered DHFR with GTP-CH activity and expression is scant. Experiments using methotrexate (MTX), an inhibitor of DHFR, did not inhibit BH_4 production in rat pineal glands, except by 30% at the highest concentration (10μM). In contrast, another study (Chalupsky *et al*, 2005) demonstrated that by inhibiting DHFR activity and expression using small interfering RNA (siRNA), BH_4 and subsequently NO decline. Furthermore, DHFR expression (Kaufman, 1991) and activity (Ludwig *et al*, 1987) are low in the brain compared to other organs, so it is possible DHFR limits BH_4 production from L-sepiapterin.

Data presented in the previous chapter (section 3.3.4), in the biosynthetic capacity bypass block experiments, would perhaps suggest that DHFR is the source of the impaired BH_4 production following L-sepiapterin treatment. These data show that when a substrate was introduced downstream of the GTP-CH metabolic block in the *hph-1* brain, the biosynthetic capacity was the same as wild type. If SR was down-regulated, then biosynthetic capacity would also be impaired. Further work for this section could be to use MTX to inhibit DHFR and compare 7,8-dihydrobiopterin production in *hph-1* and wild type cells. Impaired levels of 7,8-dihydrobiopterin would imply SR activity is attenuated, and normal levels would point toward DHFR.

4.5.5. Effect of the combination of Lipopolysaccharide and Interferon-γ with l-sepiapterin on *hph-1* and wild type astrocyte tetrahydrobiopterin and nitrite and nitrate levels

Although L-sepiapterin combined with LPS + IFN-γ did not significantly increase *hph-1* astrocyte NO_2^- and NO_3^- above *hph-1* LPS + IFN-γ stimulated levels, differences between *hph-1* and wild type stimulation were no longer apparent. Furthermore, L-sepiapterin alone had no effect on NO_2^- and NO_3^- production in either cell type, and adding L-sepiapterin to wild type cells stimulated with LPS + IFN-γ did not increase NO_2^- and NO_3^- further. This suggests wild type cells have

sufficient BH_4 to support LPS + IFN-γ stimulated NO generation, whereas supplementing *hph-1* astrocytes with L-sepiapterin is required to produce levels of NO_2^- and NO_3^- approaching wild type. One possible reason that the difference between wild type LPS + IFN-γ and *hph-1* LPS + IFN-γ + L-sepiapterin treatments did not reach significance is that BH_4 was still below wild type stimulated levels. Therefore higher concentrations of L-sepiapterin may give rise to increased NO_2^- and NO_3^-. These results provide further evidence that iNOS stimulation and subsequent NO_2^- and NO_3^- production is dependent on BH_4 levels.

L-sepiapterin is reported to enhance cytokine stimulated NO_2^- and NO_3^- in a variety of cells, including vascular smooth muscle (Gross and Levi, 1992, Nakayama *et al*, 1994, Walter *et al*, 1996), cancer lines (Kwon *et al*, 2004) and astrocytes (Miljkovic *et al,* 2002). Moreover, L-sepiapterin can support nitrite generation in LPS + IFN-γ stimulated cells, when BH_4 production is inhibited by DAHP (Hattori and Gross, 1993). L-sepiapterin supplementation in *hph-1* astrocytes may assist dimerisation of iNOS and arginine binding, or even arginine uptake into the cell. All these potential factors are discussed in section 4.5.2.

Addition of L-sepiapterin to wild type and *hph-1* astrocytes treated with LPS + IFN-γ did not appear to increase BH_4 in cells treated with L-sepiapterin or LPS + IFN-γ alone. In *hph-1* cells BH_4 concentrations remained at L-sepiapterin treated levels, whereas wild type BH_4 did not differ from LPS + IFN-γ stimulated. L-sepiapterin may not increase BH_4 in LPS + IFN-γ stimulated astrocytes because L-sepiapterin and LPS + IFN-γ produce BH_4 through a common source, i.e. SR. Substrates from the two different sources (6-pyruvoyl-tetrahydropterin and L-sepiapterin) have to compete for binding at SR, thus this may not result in greater BH_4 production. In addition BH_4 produced via LPS + IFN-γ in *hph-1* astrocytes only doubles basal levels and is not likely to add significantly to that produced from L-sepiapterin.

These results may also provide useful insight for future experiments, where adenoviral transfections will be used to correct the impaired NO status of *hph-1* astrocytes. By showing it is possible to augment stimulated NO_2^- and NO_3^- levels by increasing BH_4 via L-sepiapterin, it may be feasible that enhanced GTP-CH expression may also lead to higher NO_2^- and NO_3^- production.

4.6. CONCLUSIONS

The results of these experiments provide additional evidence of impaired BH_4 and NO status in *hph-1* astrocytes. Basal levels of BH_4 are attenuated in *hph-1* cells, although under basal conditions NO_2^- and NO_3^- are not. Stimulation of the astrocytes by LPS + IFN-γ, which causes parallel induction of GTP-CH and iNOS expression, lead to increases in BH_4 and NO_2^- and NO_3^- in wild type and *hph-1*, however in both cases this is impaired in *hph-1* cells. *hph-1* astrocytes also produce less BH_4 when responding to L-sepiapterin, which is a substrate for the pterin salvage pathway. This may suggest that the impairment of GTP-CH in these cells may lead to secondary effects on other enzymes in the salvage pathway, *i.e.* SR or DHFR. L-sepiapterin in combination with LPS + IFN-γ stimulation lead to an upregulation of NO_2^- and NO_3^- levels in *hph-1* cells, to values approaching wild type. These data suggest that transfecting *hph-1* astrocytes with an adenoviral vector containing the gene for GTP-CH may be a viable approach for correcting both the BH_4 and NO status of these cells.

Chapter 5

Gene transfer in the *hph-1* mouse astrocytes

5.1 INTRODUCTION

5.1.1. Principles and history of gene therapy

The principle of gene therapy is to use DNA as a drug to ameliorate human disease. Gene therapy aims to insert therapeutic genes into an individuals cells or tissues to treat these disorders. Typically this involves supplementing pathological mutant alleles with functional ones, but can also involve other methods such as silencing genes that are being over-expressed, and introducing cytotoxic genes to kill cancer cells (Brooks, 2002). Viruses are the most common vectors used to deliver genes to their target site, however other approaches exist that are discussed in section 5.1.4.

The benefits that gene therapy may present are significant. It offers potential to cure the patient instead of treating symptoms. A number of diseases result directly from genetic origins, such as Duchenne muscular dystrophy (DMD) and Huntingdon's chorea, whilst a vast number of others involve some genetic component, usually via up- or down-regulation of genes with pathological consequences (SoRelle, 2000). Therefore the scope of disorders that gene therapy has the potential to treat is very broad. Gene therapy has been suggested as an approach to treat diseases such as cancer (Zhang, 1999), vascular disease (Jones and Koch, 2005), cystic fibrosis (Parsons, 2005), PD (During *et al*, 2001), AIDS and other infectious diseases (Bunnell and Morgan, 1998), to name but a few. The technology is still in its infancy but has already offered successes, as well as publicised setbacks.

One of the earliest suggestions of using genetic material therapeutically was in 1968 by Rogers and Pfuderer, who proposed that viruses could be modified, then used to "transmit...information" (Rogers and Pfuderer, 1968). The first gene therapy trial in humans was conducted on two patients with thalassaemia in 1980, although in secret and without official approval (Grosshans, 2000). The first sanctioned human gene therapy study was in 1989, however the aim of this trial was not treatment but to demonstrate the safety of viral gene transduction (Rosenberg *et al*, 1990). This was followed in 1991 by a study treating two patients with a form of the genetic disease severe combined immunodeficiency (SCID), caused by the lack of functional adenosine deaminase (ADA)(Culver *et al*, 1991). Initial reports indicated success, however the gene therapy failed long term and subjects resorted back to

pharmacological intervention (Blaese *et al*, 1995; Cavazzana-Calvo *et al*, 2001). Cavazzana-Calvo and colleagues described the first successful gene therapy in humans in 2000, in patients suffering from an x-linked form of SCID. Haematopoietic cells were transfected *ex vivo* with a retrovirus expressing a γc cytokine receptor subunit, and reinfused into the patient, resulting in correction of the disease phenotype (Cavazzana-Calvo *et al*, 2000).

However, gene therapy remains controversial. The success of the cure for SCID in 2000 was tempered by the news that two of the children developed a leukaemia-type disorder, as a direct result of the treatment (Hacein-Bey-Abina *et al*, 2003). Furthermore in 1999, Jesse Gelsinger an 18-year-old suffering from a partial deficiency of ornithine transcarbamylase (OTC), was given an adenovirus as part of a study to test the safety of the vector and died 4 days later following liver failure, as a direct consequence of the treatment (Marshall, 1999).

Gene therapy clinical trials and research into more effective and safer vectors continues. At the time of writing 1192 clinical trials were completed, ongoing or pending (http://www.wiley.co.uk/genmed/clinical/). A literature search of the term "gene therapy" revealed around 2500 publications, at a rate of 130 per week in the year 2006 alone (http://www.ncbi.nih.gov/entrez/query.fcgi). Gene therapy has potential as a method of targeting the cause of the disease, rather than merely treating the symptoms, which makes this strategy an attractive proposal.

5.1.2. Methods of gene therapy

Gene therapy falls principally into two categories: somatic and germline. Somatic gene therapy is the treatment of any cell except the germline, and its purpose is to ensure treatment affects one generation and is not passed on to future progeny (Grosshans, 2000). The overall genetic makeup of the individual is not altered. So far all clinical trials currently approved in humans have been somatic gene therapy. In contrast, in germline gene therapy the sperm and ova are targeted, therefore changes in the genetic make-up are passed onto future generations. This has the potential to treat genetic diseases that may arise in unborn progeny, but is currently untested.

The two main approaches for gene therapy are *in vivo* and *ex vivo*.

5.1.2.1. *Ex vivo gene therapy*

In this approach, cultured cells obtained from a patient are transfected with a therapeutic gene, then transplanted back into the individual. The modified cells act as a "sink", producing and releasing therapeutic factors into the local environment. The cells removed must be easy to obtain and maintain in culture, accept and express the therapeutic gene, and not elicit a host immune response when re-introduced. Advantages of this approach are that cells can be characterised before genetic modification, and used only once alteration has taken place, with uninfected cells being selected out before transplantation (Selkirk, 2004). The disadvantages are difficulty of harvesting cells and maintaining them in culture, plus effective transfection of genetic material. Reintroduction may also cause immunological consequences (Selkirk, 2004). Types of cells used for this approach include primary astrocytes (Selkirk *et al*, 2002), which have so far only been used in proof of concept preclinical studies, and fibroblasts for AD (Horellou *et al*, 1990; Tuszynski, 2002, Tuszynski *et al*, 2005).

5.1.2.2. *In vivo gene therapy*

This strategy involves direct delivery of DNA into the target tissue. Requirements for this method are that the target cells are easily accessible and readily take up, integrate and express the genetic material, and infection does not spread to surrounding tissue. Viral vectors are most commonly used for *in vivo* and *ex vivo* gene therapy, however they are not the only methods of delivering genetic material. Each method of gene delivery has advantages and disadvantages, some of which are discussed below.

5.1.3. Methods of gene delivery

5.1.3.1. *Viral Vectors*

Viral vectors are useful for gene therapy as they have evolved to transmit genetic information, and use a host to carry their genome. These vectors are powerful means of delivering therapeutic genes into target, and currently have been used in approximately 70% of clinical trials (http://www.wiley.co.uk/genmed/clinical/). They are used both *in vivo* and *ex vivo* (Brooks, 2002), and must be modified for clinical use. Modifications are necessary to prevent viral replication, so they do not multiply and become actively pathogenic. Viral genes may also initiate pathogenic host response, so these must be removed along with non-essential genes, to allow

insertion of therapeutic genetic material. All viral vectors have both advantages and disadvantages to their use. Some of these vectors are described below.

5.1.3.2 *Adenoviral delivery vectors*

Currently 26% of gene therapy clinical trials have used adenoviral vectors (http://www.wiley.co.uk/genmed/clinical/), making them the most common vector in gene delivery. Adenoviruses are non-enveloped double-stranded DNA viruses usually associated with respiratory infections in humans, but may also cause gastrointestinal and ocular complaints (Vargosko *et al*, 1965). There are at least 51 serotypes of adenovirus, classified into 6 subgroups A-F. Serotype 5 (Ad5) and serotype 2 (Ad2), which belong to subgroup C, have been used most extensively in gene therapy (Xu *et al*, 2005). They infect dividing and non-dividing cells readily, and most cells are susceptible to infection. Entry of the adenovirus into the host cell occurs by receptor-mediated endocytosis, via the coxsackievirus and adenovirus receptor (CAR), a widely distributed membrane protein (Roelvink *et al*, 1998). Once inside the cell the virus is packaged inside an endosome and avoids lysosomal degradation. Inside the endosome the virus is disassembled, allowing the virus to release its genome into the nucleus (Xu *et al*, 2005), whereupon the host's nuclear machinery replicates the virus (figure 5.1). Adenoviral DNA is not integrated into the host genome, which means that transfection is transient. In gene transfer therapeutic DNA takes the place of viral DNA, and beneficial proteins are generated in place of viral replication.

Non-integration of the adenoviral DNA into the host genome has advantages and disadvantages. Short-term expression is beneficial in treating disorders like cancer, where therapeutic genes may not be useful once the disease is treated (Stewart *et al*, 1999). However problems arise treating chronic diseases such as cystic fibrosis, which require repeated administration of adenovirus. This presents major difficulties, as one of the main setbacks of adenoviruses is the severe innate immune response they can trigger (Kafri *et al*, 1998). Indeed, this property limits their survival in host cells and most hinders their use in gene therapy (Byrnes *et al*, 1995; van Ginkel *et al*, 1997). As a result these viral vectors have been extensively engineered and toxicity has been attenuated through the development of helper-dependent adenoviruses (HD-

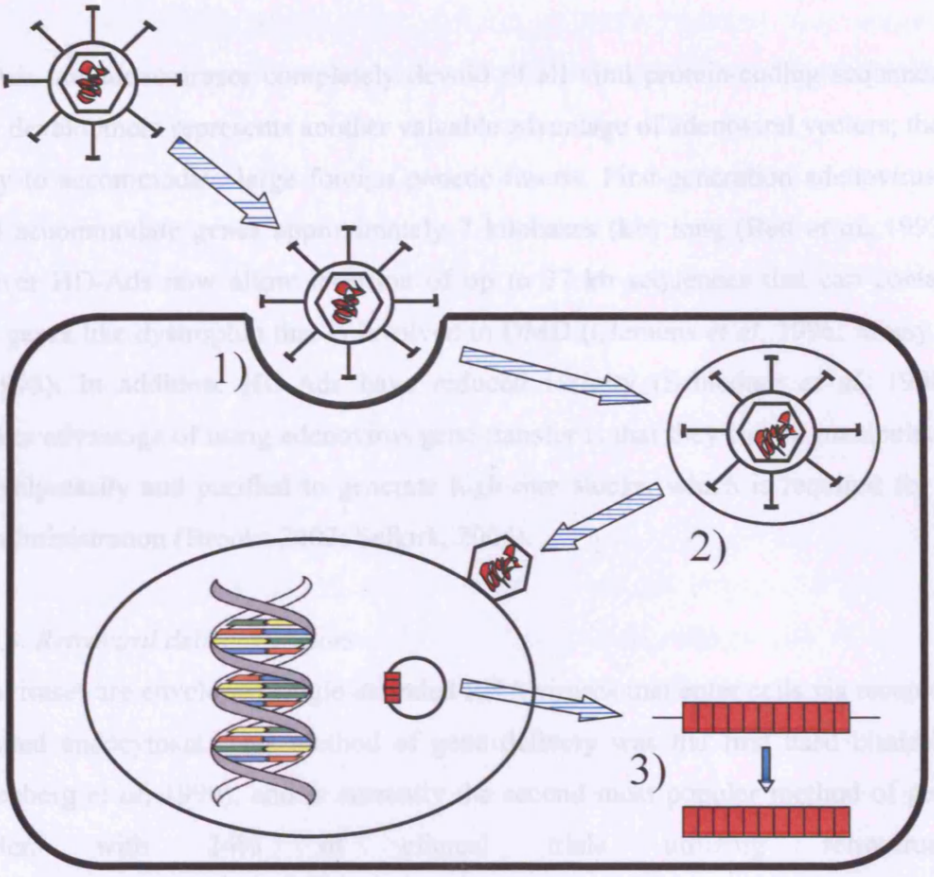


Figure 5.1. Adenoviral-mediated gene delivery. Step 1); Adenoviral vector binds and enters the cell via receptor-mediated endocytosis. Step 2); Adenovirus is released from the endosome and the viral capsid is translocated into the nucleus. Step 3); Therapeutic DNA is transcribed and translated from episomal adenoviral genome, resulting in beneficial protein.
Adapted from Brooks, 2002.

Ads), which decrease immunogenicity, and prolong the life of the virus making them more useful long-term (Morsy and Caskey, 1999).

HD-Ads are adenoviruses completely devoid of all viral protein-coding sequences. Their development represents another valuable advantage of adenoviral vectors; their ability to accommodate large foreign genetic inserts. First-generation adenoviruses could accommodate genes approximately 7 kilobases (kb) long (Bett *et al*, 1993), however HD-Ads now allow insertion of up to 37 kb sequences that can contain large genes like dystrophin that is involved in DMD (Clemens *et al*, 1996; Morsy *et al*, 1998). In addition, HD-Ads have reduced toxicity (Schiedner *et al*, 1998). Another advantage of using adenovirus gene transfer is that they can be manipulated relatively easily and purified to generate high-titre stocks, which is required for *in vivo* administration (Brooks 2002; Selkirk, 2004).

5.1.3.3. *Retroviral delivery vectors*

Retroviruses are enveloped single-stranded RNA viruses that enter cells via receptor-mediated endocytosis. This method of gene delivery was the first used clinically (Rosenberg *et al*, 1990), and is currently the second most popular method of gene transfer, with 24% of clinical trials utilizing retroviruses (http://www.wiley.co.uk/genmed/clinical/). Once inside the host cell, viral RNA is reverse-transcribed to DNA and then integrated into the host genome. The gene inserted will then be maintained for the life of the cell, and will be part of the resulting daughter cells should replication take place. This leads to long-term expression of retroviral DNA, and makes it a good candidate for gene delivery, with *in vivo* application established in several diseases including SCID (section 5.1.1)(Cavazzana-Calvo *et al*, 2000) and ovarian cancer (Tait *et al*, 1999).

Retroviruses can incorporate therapeutic genes into the cell efficiently, however they integrate randomly into the host genome. This can be disadvantageous if viral DNA is inserted into an essential gene, causing loss of function and even tumourigenesis in some cases (Check, 2002). Additionally, retroviruses are unable to infect non-dividing cells, so gene therapy can only be targeted at tissues where cell replication occurs (Roe *et al*, 1993; Lewis and Emerman, 1994), although this maybe useful for tumour therapy (Wu and Ataai, 2000). Furthermore, there are restrictions of

approximately 8 kb on the size of therapeutic genes (Shin *et al*, 2000), so larger genes cannot be delivered via retrovirus. Additionally retroviral vectors are difficult to produce in high-titre stocks (Wu and Ataai, 2000). However they are well characterised and a relatively safe, and remain popular in gene therapy clinical trials.

5.1.3.4. *Other types of viral vector*

Adenoviruses and retroviruses are the most popular gene therapy vectors, however others exist that may offer improved delivery, and make up approximately 20% of trials. Lentiviruses are a class of retroviruses that includes human immunodeficiency virus (HIV) and can transfer genes to non-dividing cells (Lewis and Emerman, 1994; Naldini *et al*, 1996). It is possible to purify lentiviral vectors to high titres, however there are safety concerns due to the HIV derivation, and the risk of re-activation.

Adeno-associated viruses (AAV) are linear single-stranded viruses not associated with any disease in humans (Rose *et al*, 1969), which reduces risk of adverse reactions (Jooss *et al*, 1998). Like adenoviruses, they infect dividing and non-dividing cells. However AAV's can integrate into the genome of infected cells at specific locations, which in humans is chromosome 19 (Samulski *et al*, 1991). Once the AAV genome is in the nucleus the single-stranded DNA is synthesized to form double stranded DNA that is incorporated into the host genome. However, the size of genes that can be inserted is currently only 5 kb, which limits their use.

5.1.4. Non-viral methods of gene therapy

Concern over the safety of viral vectors for gene therapy has lead to the development of non-viral vectors as alternatives. Generally these are safer and easier to produce in large quantities than viruses, however they also have some disadvantages. Some of these methods used are described below.

5.1.4.1. *Naked DNA*

This method involves the direct transfer of therapeutic DNA incorporated in a plasmid vector into the host, and can be done *in vivo* or *ex vivo* like viral transfection. In *ex vivo* naked DNA transfer, patients cells are removed and have therapeutic DNA inserted, usually by microinjection. Expression of the therapeutic gene is confirmed before the cells are implanted back into the patient. This method has been used

successfully in endothelial and smooth muscle cells (Nakamura *et al*, 1998; Mann *et al*, 1999), although it is limited in cells types that are difficult to harvest or maintain.

In vivo naked DNA delivery is the simplest of all gene therapy methods, as the therapeutic genetic material is injected straight into the tissue. It was first used in 1990 in a mouse model, and use continues today in patients being treated for conditions including critical limb ischaemia (Isner, 1998; Nakagami *et al*, 2005), and myocardial ischaemia (Losordo *et al*, 1998). The exact mechanism of uptake is unknown, but DNA can be introduced in a variety of ways including intramuscular injection, inhalation and intravascular delivery (Wolff *et al*, 1990). Cellular uptake of naked DNA is inefficient, however techniques such as the particle bombardment or "gene gun" approach, and electroporation, can increase uptake. The gene gun approach uses gold or tungsten particles coated with therapeutic DNA, propelled at high velocity using a pulse of helium gas into the cell (Fynan *et al*, 1993). Electroporation involves high-voltage electrical pulses that disrupt cellular membranes to deliver DNA. However a large number of cells die in this method, which has been minimised *in vivo* (Rols *et al*, 1998). This has been successful in skin and muscle (Rols *et al*, 1998). So far naked DNA gene therapy has been used in 17% of all gene therapy clinical trials (http://www.wiley.co.uk/genmed/clinical/).

5.1.4.2. *Cationic lipids and polymers*

Cationic lipids and polymers can transfer DNA into cells when the tissues being treated are inaccessible to naked DNA. Lipids or polymers are mixed with the DNA to form lipid/DNA complexes that can enter cells by endocytosis. Once inside the cell the DNA is released and must travel to the nucleus to be expressed. This method has been successful in delivering the cystic fibrosis transmembrane conductance regulator (CFTR) gene to cystic fibrosis patients (Noone *et al*, 2000) and to patients with melanoma (Nabel *et al*, 1996). This form of gene transfer accounts for 8% of clinical trials (http://www.wiley.co.uk/genmed/clinical/).

5.1.5. Gene therapy in neurodegenerative disease

Neurodegenerative diseases such as AD and PD respond little to treatment and currently have no cures. In addition there are long-term problems associated with drug treatment for these disorders. Gene therapy may be beneficial in

neurodegenerative diseases as a more direct, curative approach. In patients suffering from AD, *ex vivo* gene therapy using fibroblasts engineered to produce nerve growth factor (NGF) resulted in an improvement in cognitive decline in phase 1 clinical trials (Tuszynski *et al*, 2005). Clinical trials involving production of the inhibitory amino acid neurotransmitter γ-aminobutyric acid (GABA) in the subthalamic nucleus via AAV gene therapy, have been approved for PD patients. This study aims to prevent over-stimulation of the globus pallidus that occurs in PD (During *et al*, 2001), and preliminary results presented at the Society for Neuroscience, 2006 suggest this may be a successful approach (During *et al*, 2006). In another phase 1 clinical trial AAV-mediated delivery of aromatic amino acid decarboxylase (AADC) to the striatum of patients with PD, aims to increase the clinical effectiveness of levodopa drug therapy, by enhancing the conversion of this precursor to DA (Dass *et al*, 2006). Early results from this trial appear to be encouraging (http://www.avigen.com/press_release/2005/Avigen_EarlyData_PDClinicalTrial_071805.php). Additionally, a study aiming to deliver neurturin (NTN), a growth factor belonging to the same family as glial cell-derived neurotrophic factor (GDNF), via an AAV vector to the striatum of Parkinson's patients, has reported a 40% improvement in motor scores (http://www.ceregene.com/press_101006.asp). In a primate model of PD, lentiviral delivery of glial cell line-derived neurotrophic factor (GDNF) prevented nigrostriatal degeneration and improved regeneration (Kordower *et al*, 2000). There are several other neurodegenerative diseases that gene therapy is being evaluated for including Huntingdon's disease (HD) and Amyotrophic lateral sclerosis (ALS), although these are still at the preclinical stage (Haque and Isacson, 1997; Mohajeri *et al*, 1999).

5.1.6. Gene therapy for tetrahydrobiopterin deficient states

Although gene therapy has not been used clinically in BH_4 deficiencies, preclinical data suggests this approach may be valuable. Introducing genes that can elevate the production of BH_4 may be useful in neurodegenerative diseases, where acquired BH_4 deficiencies are observed, and in inborn errors of metabolism where a lack of BH_4 is fundamental to the disease state.

5.1.6.1. *Gene therapy for tetrahydrobiopterin deficiencies in neurodegenerative disease*

Gene transfer using GTP-CH transfection, combined with genes regulating DA production such as tyrosine hydroxylase (TH), partially restores behavioural and biochemical deficits in animal models of PD (Bencsics *et al*, 1996; Leff *et al*, 1998; Mandel *et al*, 1998). Intrastriatal injection of 6-hydroxydopamine (6-OHDA) induces a PD-like syndrome in rats, which can be ameliorated using both *ex vivo* and *in vivo* AAV-mediated gene transfer of human TH and GTP-CH. This increases levels of L-Dopa in the rat striatum, resulting in an improved behavioural profile (Bencsics *et al*, 1996; Leff *et al*, 1998; Mandel *et al*, 1998). Furthermore, a study involving triple AAV-mediated transduction of the striatum in 6-OHDA treated Parkinsonian rats, showed that gene transfer with TH, AADC and GTP-CH could increase DA levels and improve the Parkinson's-like behavioural syndrome, above values reported in rats transfected with just TH and AADC (Shen *et al*, 2000).

5.1.6.2. *Gene therapy for inborn errors of tetrahydrobiopterin metabolism*

In human fibroblasts derived from PTPS-deficient patients, retroviral gene transfer of GTP-CH, PTPS and SR increases BH_4 dramatically, demonstrating a use for gene transfer in these disorders (Laufs *et al*, 1998, 2000). Amplifying BH_4 using viral transfection can also augment NOS function. An adenovirus encoding GTP-CH raised BH_4 levels, as well as NOS activity, protein and dimerisation in human endothelial cells (HEC's)(Cai *et al*, 2002). Furthermore, when BH_4 was increased in hyperglycaemic HEC's using an adenovirus containing GTP-CH, NO production was augmented and O_2^- production decreased (Cai *et al*, 2005). Endothelial cells and arterial rings from a rat model of diabetes had impaired NO synthesis. NO production and vasorelaxation could be improved when BH_4 levels were elevated with adenoviral GTP-CH treatment (Meininger *et al*, 2004). Attenuated NOS function is also observed in diabetic (Alp *et al*, 2003), hypertensive (Zheng *et al*, 2003) and atherosclerotic (Alp *et al*, 2004) rats *in vivo,* which can be reversed when gene transfer is used to increase BH_4. Taken together these data demonstrate the amelioration of some BH_4-deficient states.

5.2. AIMS

- Use adenoviral mediated gene transfer with AdGCH to introduce human GTP-CH into *hph-1* astrocytes, to correct the defect in BH_4 metabolism in these cells.
- Confirm successful adenoviral gene transfer infection and expression in *hph-1* astrocytes using confocal microscopy and Western blotting.
- Investigate the ability of AdGCH to correct impaired NO metabolism in *hph-1* astrocytes, using NO_2^- and NO_3^- measurements as an index of NO production, under basal and LPS + IFN-γ stimulated conditions.
- Investigate the molecular mechanisms behind the impaired NO metabolism found in *hph-1* astrocytes after LPS + IFN-γ stimulation, by using Western blotting techniques to measure the total amount of iNOS protein expressed and the relative levels of iNOS dimer and monomer found in the cell.
- Use AdGCH transfection to attempt to correct any defects in iNOS protein expression or dimer formation found in *hph-1* astrocytes following LPS + IFN-γ stimulation.

5.3. METHODS

5.3.1. Adenoviral construction

All work involved in the construction of adenoviral vectors was carried out by Dr. Shijie Cai at the Department of Cardiovascular Medicine, at the John Radcliffe Hostpital, University of Oxford according to the method described in Cai *et al*, 2002. Briefly, 1.2-kb human GTP-CH cDNA (provided to Dr. Cai by Dr. H Ichinose, Japan) (Togari *et al*, 1992) was modified using polymerase chain reaction (PCR) and subcloned to include a haemagglutinin (HA) epitope tag at the 5' end (figure 5.2 B). This HA-tagged human GTP-CH cDNA was then cloned into the plasmid pShuttleCMV (provided to Dr. Cai by Dr. Bert Vogelstein) (He *et al*, 1998), and used to generate a recombinant adenovirus AdGCH. This adenovirus encodes HA-GTP-CH under the control of a cytomegalovirus immediate-early promoter, and was produced by transfection in 293 cells using the AdEasy system (He *et al*, 1998). A recombinant adenovirus AdeGFP was generated using the same system (Cai *et al*, 2005) and used as a control for viral infection (Cai *et al*, 2005). Viruses were isolated by three rounds of plaque purification, amplified in 293 cells, then purified using CsCl gradient ultracentrifugation (Channon *et al*, 1996). The structure of the human *GCH* gene in AdGCH (Togari *et al*, 1992), and a schematic outline of AdEasy system used to generate AdGCH and AdeGFP (He *et al*, 1998) are shown in figure 5.2 C.

5.3.2. Cell culture

Primary astrocytes were cultured from wild type and *hph-1* mouse neonates as described in section 2.2.2. On day 13 cells were transported to the Wellcome Trust centre for Human genetics, Department of Cardiovascular Medicine, at the John Radcliffe Hostpital, University of Oxford, to undergo gene transfer. For transportation to Oxford cell culture flasks were filled to capacity with MEM supplemented with 1% volume/volume antibiotic antimycotic, along with 10% FBS (vol/vol) and 2mM L-Glutamine, and placed in an insulated environment. Transportation time varied, but was typically between 2.5 to 3 hours. Upon reaching Oxford, media was replaced by fresh MEM, and cells placed into an incubator at 37°C, with 5% CO_2, 95% air and 95% humidity. After 2 hours cells were passaged

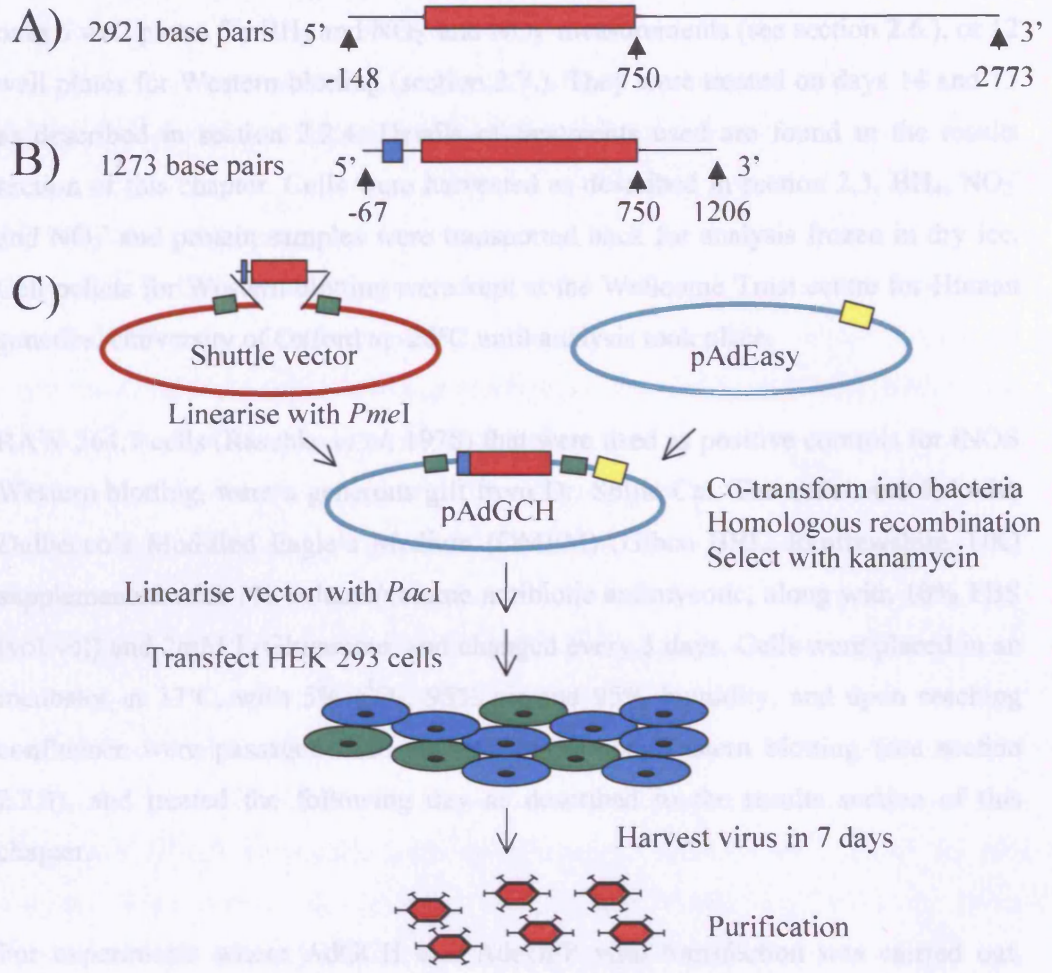


Figure 5.2. A) The structure of the full-length cDNA for human GTP-cyclohydrolase type 1 (*GCH*). B) The structure of human *GCH* type 1 used by Cai *et al* (2002) in AdGCH. Line, untranslated region; red box, coding region; blue box, 5' haemagluttinin tag. C) Simplified schematic outline of the AdEasy system for producing AdGCH and AdeGFP (Cai *et al*, 2001). *GCH* (or *eGFP*) is cloned into a shuttle vector under the control of a cytomegalovirus immediate-early promoter (green boxes). The plasmid is linearized by digesting with restriction endonuclease *Pme*I, and cotransformed into *E. coli* cells with an adenoviral backbone plasmid (pAdEasy) containing gene for kanamycin resistance (yellow box). Recombinants (pAdGCH) are selected for kanamycin resistance, and recombination was confirmed by multiple restriction endonuclease analyses. The linearized (*Pac*I) recombinant plasmid is then transfected into adenovirus HEK 293 packaging cell lines, which produce recombinant adenoviruses that are typically generated within 7–10 days. Viruses are purified with CsCl gradient ultracentrifugation (Channon *et al*, 1996).

onto 6 well plates for BH_4 and NO_2^- and NO_3^- measurements (see section 2.6.), or 12 well plates for Western blotting (section 2.7.). They were treated on days 14 and 15 as described in section 2.2.4. Details of treatments used are found in the results section of this chapter. Cells were harvested as described in section 2.3. BH_4, NO_2^- and NO_3^- and protein samples were transported back for analysis frozen in dry ice. Cell pellets for Western blotting were kept at the Wellcome Trust centre for Human genetics, University of Oxford at -20°C until analysis took place.

RAW 264.7 cells (Raschke *et al*, 1978) that were used as positive controls for iNOS Western blotting, were a generous gift from Dr. Shijie Cai. The cells were fed with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Renfrewshire, UK) supplemented with 1% volume/volume antibiotic antimycotic, along with 10% FBS (vol/vol) and 2mM L-Glutamine, and changed every 3 days. Cells were placed in an incubator at 37°C, with 5% CO_2, 95% air and 95% humidity, and upon reaching confluence were passaged onto 12 well plates for Western blotting (see section 2.2.3), and treated the following day as described in the results section of this chapter.

For experiments where AdGCH and AdeGFP viral transfection was carried out, viruses were kindly donated by Dr. Shijie Cai. AdGCH and AdeGFP were supplied in known titres, and then diluted accordingly. Stock titres of viruses were in plaque forming units (pfu), which are units of infectious virus that determine the ability of the virus to form a "plaque", or area of lysed cells on a monolayer of susceptible cells. This indicates how many particles are present in a stock solution. Multiplicity of infection units (MOI) are then calculated by dividing the number of phage added (pfu) by the number of cells present. This gives a measure of the number of viral particles added per cell. So 1.0 MOI indicates 1 viral particle per cell.

5.3.3. Tetrahydrobiopterin measurement

Cells were prepared for analysis of BH_4 and measured as described in section 2.3.4 and section 2.1 respectively.

5.3.4. Nitrite and nitrate measurement

NO_2^- and NO_3^- were measured in the cell culture media of wild type and *hph-1* astrocytes as an index of NO production, which is described in section 2.6.

5.3.5. Western blotting

Western blotting was carried out as described in section 2.7. To investigate iNOS homodimer formation in endothelial cells, low-temperature SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described previously (Klatt *et al*, 1995). Throughout the preparation for Western blotting cells were kept on ice to prevent dimer dissociation. 10μg of cellular protein was run at 5mA overnight on ice to keep temperature to a minimum, and then transferred for 3 hours at 400mA. For primary antibody binding each membrane was incubated at 4°C overnight with 20ml of a 1:2000 dilution of mouse anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) in 6% weight:volume Marvel ® (Premier International Food) in PBS-T. For the secondary antibody membranes were incubated with 20 ml of a 1:4000 dilution of polyclonal anti-mouse horse-radish peroxidase (HRP) conjugate antibody (Promega, Madison WI, USA) in 6% weight:volume Marvel ® (Premier International Food) in PBS-T at room temperature for an hour. Bands were visualised using chemiluminescence, and quantified using GelPro analysis system and software (Media Cybernetics, Silver Springs, MD). Protein bands of approximately 130kDa and 260kDa were measured as iNOS monomer and dimer, respectively (Geller *et al*, 1993).

For total iNOS Western blots, samples were first heated at 60°C for 5 minutes to ensure that iNOS protein was predominantly as the 130kDa monomer form. 7μg of protein from each sample was run for 1.5 hours at 100mA, and transferred for 3 hours at 400mA. Primary and secondary antibody incubations were the same as described above for iNOS homodimer formation. A band at 130kDa was quantified as total iNOS (Geller *et al*, 1993).

Western blotting for recombinant GTP-CH protein expressed in *hph-1* astrocytes was performed in the same manner as for total iNOS. Samples were heated at 60°C for 5 minutes then 20μg of protein was run for 1.5 hours at 100mA, and transferred for 1.5 hours. Recombinant human GTP-CH cloned into AdGCH had been subcloned to

contain a HA epitope tag. Anti-HA antibodies were used to visualize recombinant GTP-CH protein in cells transfected with AdGCH. For primary antibody incubations, membranes were left overnight at 4°C with 20 ml of 1:1000 mouse anti-HA high affinity monoclonal (Roche diagnostics, Lewes, UK) in 6% weight:volume Marvel ® (Premier International Food) in PBS-T. Secondary antibody incubations were performed with 20ml 1:2000 anti-mouse HRP conjugate antibody (Promega, Madison WI, USA) in 6% weight:volume Marvel ® (Premier International Food) in PBS-T. Bands visualised at approximately 30kDa were determined to be recombinant GTP-CH protein (Cai et al, 2002). As a positive control, cell lysates from endothelial cells transfected with 50 multiplicity of infection units of AdGCH, kindly donated by Dr. Shijie Cai were included in the experiment.

Western blotting for GAPDH was also carried out on membranes that had been used for total iNOS protein semi-quantification, to show equal protein loading. GAPDH is often used to demonstrate equal protein loading, as it is ubiquitously expressed (Zhang *et al*, 2004; Ferguson *et al*, 2005). However, NO production can inhibit GAPDH activity (Padgett and Whorton, 1995), so it was recognized that results may not show equal intensity bands. It was proposed that β-actin, another ubiquitously expressed protein, may be used in place of GAPDH. However it was not possible to visualise any β-actin protein using these samples, possibly due to the low quantity of protein loaded (7μg). Membranes that had previously been used for total iNOS Western blots were stripped with a solution of 6.25mM Tris pH 6.8 containing 2% SDS and 100mM β-mercaptoethanol, to remove any associated antibodies bound to the membrane. Membranes were then incubated with 20ml of 1:1500 mouse primary anti-GAPDH monoclonal antibody (Chemicon, Temecula, CA USA), followed by 20ml 1:2000 anti-mouse HRP secondary conjugate antibody (Promega). Bands of 35kDa size were determined to be GAPDH.

5.3.6. Confocal microscopy

Visualisation of cells expressing recombinant eGFP from AdeGFP was demonstrated using confocal microscopy, which is described in section 2.8.

5.3.7. Statistical analysis

Statistical tests were conducted as described in section 2.10.

5.4. RESULTS

5.4.1. Basal tetrahydrobiopterin and NO_2^- and NO_3^- levels in wild type and *hph-1* mouse astrocytes used for 24 hours treatments

Table 5.1 shows that basal BH_4 levels in wild type astrocytes used for 24-hour treatments remain significantly lower in *hph-1* astrocytes compared to wild type ($p < 0.05$, n = 5-7). BH_4 levels in the *hph-1* astrocytes were approximately 51% of wild type. This demonstrates that after transporting the cells to Oxford, a BH_4 deficiency was still observed in the *hph-1* astrocytes.

Corresponding basal levels of NO_2^- and NO_3^- were not significantly different between wild type and *hph-1* astrocytes ($p = 0.36$, n = 4-5) (table 5.2).

5.4.2. Effect of AdGCH adenoviral transfection on tetrahydrobiopterin and NO_2^- and NO_3^- levels in *hph-1* astrocytes

Table 5.1 shows *hph-1* astrocytes transfected for 24 hours with 200, 10, 5, 2.5, 0.2 and 0.1 multiplicity of infection (MOI) units of AdGCH had increased levels of BH_4 that rose in a dose related manner, except at the highest dose 200 MOI where a degree of cell death occurred. The dose response curve was the product of attempts to modify the amount of BH_4 generated to appropriate levels. A dose of 0.1 MOI was selected for most experiments (unless specified), as this is within the range of wild type LPS + IFN-γ stimulated BH_4 levels. AdGCH at 0.1 and 0.2 MOI significantly increased BH_4 levels above basal ($p < 0.01$ and 0.001, respectively; n = 6-10). BH_4 increased approximately 80-fold in 0.2 MOI and 50-fold in 0.1 MOI AdGCH treated astrocytes. Control virus AdeGFP has no effect on basal BH_4 levels, at any MOI tested (data not shown).

However, adenoviral transduction with 0.2 MOI or 0.1 MOI AdGCH had no significant effect on basal NO_2^- and NO_3^- in *hph-1* astrocytes ($p > 0.05$, n = 6-7). 0.1 MOI AdeGFP also did not significantly affect basal NO_2^- and NO_3^- ($p > 0.05$, n = 6-7) (table 5.2). Doses of 5.0 and 2.5 MOI also appeared to have no effect on basal NO_2^- and NO_3^- (table 5.2).

Treatment	BH_4 pmol/mg protein
WT Control	7.35 ± 1.28
hph-1 control	3.22 ± 0.36 †
hph-1 0.1 MOI AdeGFP	3.10 ± 0.60 †
hph-1 0.1 MOI AdGCH	197.39 ± 37.02 **
hph-1 0.2 MOI AdeGFP	2.93 ± 0.60 †
hph-1 0.2 MOI AdGCH	313.69 ± 61.57 *
hph-1 2.5 MOI AdGCH	1004.20 (n = 1)
hph-1 5.0 MOI AdGCH	1617.74 (n = 1)
hph-1 10.0 MOI AdGCH	8017.68 (n = 1)
hph-1 200 MOI AdGCH	457.31 (n = 1)

Table 5.1. BH_4 levels in wild type (WT) and *hph-1* astrocytes following adenoviral transfection.

Astrocytes were incubated in the presence and absence of 200, 10, 5, 2.5, 0.2 and 0.1 multiplicity of infection units (MOI) AdGCH and AdeGFP. Cells were harvested after 24 hours and BH_4 was measured in extracts of homogenised cells by reverse-phase HPLC coupled with ECD. Data expressed as mean ± SEM of 3-10 independent cell preparations (except where indicated). †, $p < 0.05$ vs. wild type untreated control determined by one way ANOVA followed by least significant difference test. *, $p < 0.01$ vs. *hph-1* untreated control, **, $p < 0.001$ vs. *hph-1* untreated control determined by one way ANOVA followed by least significant difference test.

Treatment	NOx nmol/mg protein
WT Control	2.37 ± 0.51
hph-1 control	2.80 ± 0.82
hph-1 0.1 MOI AdeGFP	1.91 ± 0.23
hph-1 0.1 MOI AdGCH	2.58 ± 0.26
hph-1 0.2 MOI AdeGFP	2.19 ± 0.73
hph-1 0.2 MOI AdGCH	2.47 ± 0.50
hph-1 2.5 MOI AdGCH	2.85 (n = 1)
hph-1 5.0 MOI AdGCH	3.41(n = 1)

Table 5.2. NO_2^- and NO_3^- levels in cell culture media of wild type (WT) and *hph-1* astrocytes following adenoviral transfection. Astrocytes were incubated in the presence and absence of 5.0, 2.5, 0.2, 0.1 multiplicity of infection units (MOI) AdGCH and 0.1 and 0.2 MOI AdeGFP. NO_2^- and NO_3^- concentrations were measured using the Griess reaction. Data expressed as mean ± SEM of 3-10 independent cell preparations (except where indicated).

5.4.3. Expression of AdGCH in *hph-1* astrocytes determined by Western blotting

Expression of the recombinant human GTP-CH protein was confirmed in *hph-1* astrocytes transfected with 0.1 and 0.2 MOI AdGCH (figure 5.3). No AdGCH expression was observed in other treatment groups. Expression of recombinant human GTP-CH protein from AdGCH appeared to increase as the titre of the virus and levels of BH_4 were enhanced, as 0.2 MOI AdGCH showed greater intensity than 0.1 MOI. As a positive control, cell lysates from endothelial cells transfected with 50 MOI AdGCH were included in the Western blot to show that the protein expressed was indeed recombinant human GTP-CH tagged with the HA epitope. These cell lysates had been used in published data demonstrating expression of GTP-CH from AdGCH (Cai *et al*, 2002). A strong band was observed in positive control lanes.

5.4.4. Visualisation of *hph-1* astrocytes transfected with AdeGFP using fluorescence microscopy

Treatment of cells with AdeGFP enables visualisation of expression of recombinant viral proteins. Confocal microscopy showed that *hph-1* astrocytes transfected with 200, 100, 10, 0.2 and 0.1 MOI AdeGFP expressed the recombinant green fluorescent protein (figure 5.4). Furthermore, as the viral titre increases, fluorescence appears to enhance. In addition, only a small proportion of cells contained fluorescence at 0.1 and 0.2 MOI, which is consistent with the doses used, where only theoretically 1 in 10 and 2 in 10 respectively of the cells are transfected. At higher concentrations of vector (10, 100, 200 MOI AdeGFP), greater dose-dependent levels of transfection are observed.

5.4.5. Effect of increasing exogenously applied tetrahydrobiopterin on intracellular tetrahydrobiopterin in wild type and *hph-1* cortical astrocytes.

Uptake of BH_4 into *hph-1* astrocytes was investigated, to find whether the BH_4 demonstrated to be produced and released by Cai *et al*, 2002 in endothelial cells following transfection with AdGCH, can be taken up by neighbouring cells, or whether the 1 in 10 cells that are theoretically transfected are the sole source of the BH_4 measured. Both wild type and *hph-1* astrocytes were included to compare uptake of cofactor between cell types. Astrocytes were incubated for 3 hours with 1, 5, 10, 20 and 50μM BH_4, washed 3 times with HBSS

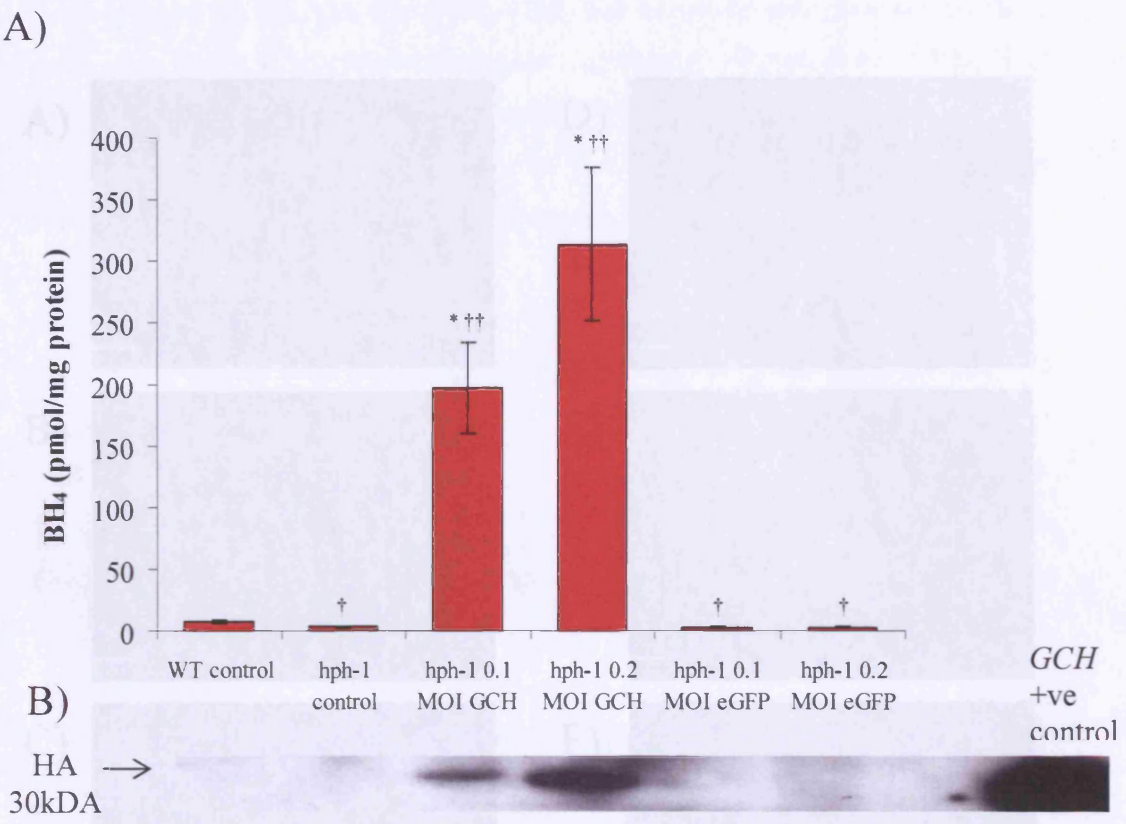


Figure 5.3. Effect of 0.1 and 0.2 multiplicity of infection units (MOI) AdGCH and 0.1 and 0.2 MOI of control virus AdeGFP on BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

A) BH_4 was measured using reverse-phase HPLC coupled with ECD. Data is expressed as mean ± SEM of 3-10 independent cell culture preparations. * = $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. † = $p < 0.05$ vs. wild-type, †† = $p < 0.01$ vs. wild-type calculated using Student's t-test.

B) Expression of recombinant human GTP-CH from AdGCH in *hph-1* astrocytes. Western blotting was carried out as described in section 2.7. Cell lysates were fractionated by SDS–PAGE and immunoblotted with antibodies to HA tag epitope. As a positive control, cell lysates from endothelial cells transfected with 50 MOI AdGCH were included in the experiment.

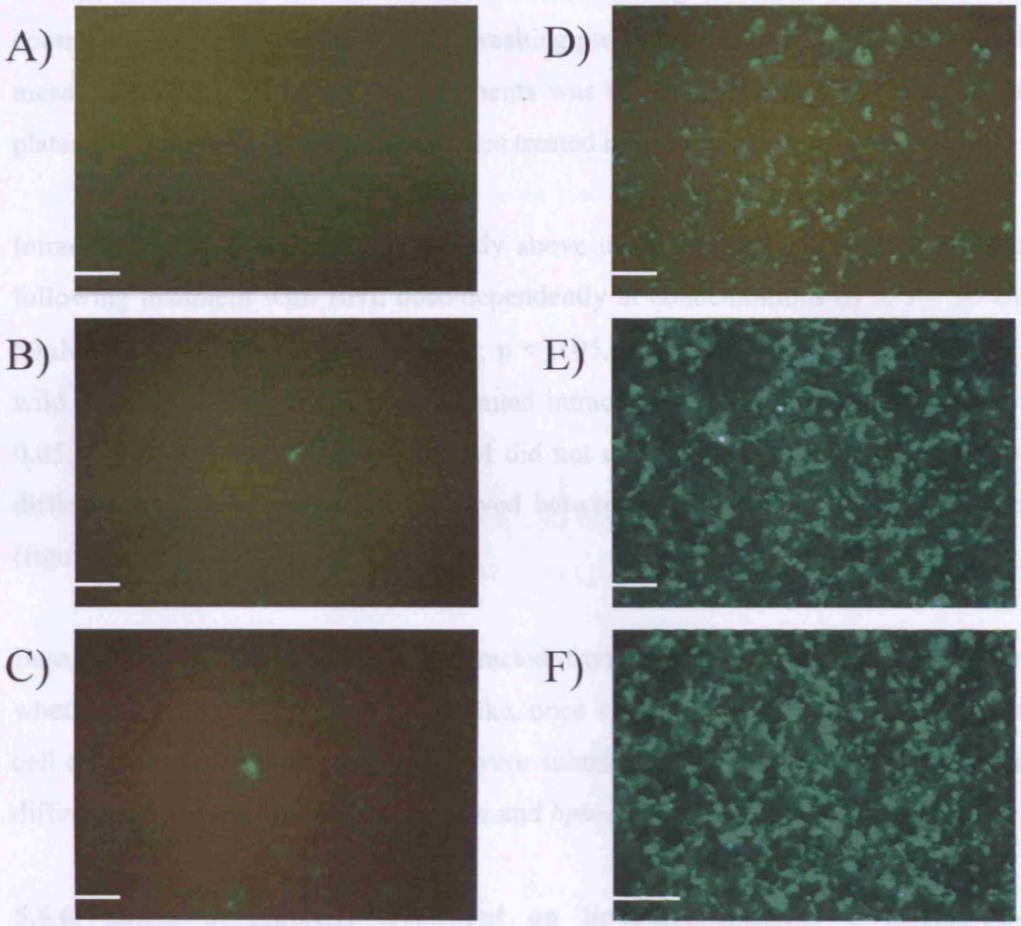


Figure 5.4. *hph-1* astrocytes treated with A) Control cells – no virus, B) 0.1 MOI AdeGFP, C) 0.2 MOI AdeGFP, D) 10 MOI AdeGFP E) 100 MOI AdeGFP and F) 200 MOI AdeGFP, visualised using confocal fluorescence microscopy. Cells expressing eGFP are visible as areas of greater fluorescent intensity, an effect that appears to be dose-dependent. These images are representative of individual cells that display the typical distribution of eGFP. Bar = 50μm

then harvested for BH_4. An empty poly-L-ornithine coated 6-well plate was included in the experiment, which was incubated with increasing concentrations of BH_4. This control measure was to show that the washing procedure was effective, and that BH_4 measured in cells from these experiments was not from residual BH_4 left on the plates. BH_4 was not detected in the plates treated in this manner (data not shown).

Intracellular BH_4 increased significantly above untreated levels in *hph-1* astrocytes following treatment with BH_4, dose-dependently at concentrations of 5, 10, 20 and 50μM ($p < 0.01$ for 5-20μM, $n = 3$-4; $p < 0.05$, $n = 3$-4 for 50μM)(figure 5.5). In wild type, exogenous BH_4 also augmented intracellular BH_4 dose-dependently ($p < 0.05$, $n = 4$-7). Concentrations of 1μM did not elevate BH_4 in either cell type. No differences in BH_4 uptake was observed between wild type and *hph-1* astrocytes (figure 5.5.).

Basal values of BH_4 were then subtracted from these concentrations, to observe whether there is any difference in uptake, once values of BH_4 already present in the cell are deducted. When basal levels were subtracted, there were still no significant differences between uptake in wild type and *hph-1* cells (table 5.3.).

5.4.6. Effect of AdGCH treatment on lipopolysaccharide + interferon-γ stimulated tetrahydrobiopterin and NO_2^- and NO_3^- levels in wild type and *hph-1* astrocytes

To assess whether adenoviral transfection with AdGCH can correct the impairment in LPS + IFN-γ stimulated NO metabolism in *hph-1* astrocytes (figure 5.6.), cells were treated with a combination of 0.1 AdGCH and LPS + IFN-γ for 24 hours. LPS + IFN-γ stimulation increased NO_2^- and NO_3^- levels in wild type and *hph-1* astrocytes, an effect that was attenuated in *hph-1* cells ($p < 0.05$, $n = 4$-5). Co-stimulation with 0.1 MOI AdGCH had no effect on NO_2^- and NO_3^- levels in *hph-1* astrocytes treated with LPS + IFN-γ, whilst 0.1 MOI AdeGFP also had no effect (figure 5.6.).

When BH_4 levels were analysed following these treatments, BH_4 was lower in *hph-1* astrocytes compared to wild type following stimulation ($p < 0.05$, $n = 4$-5). The combination of 0.1 MOI AdGCH with LPS + IFN-γ stimulation in *hph-1* astrocytes,

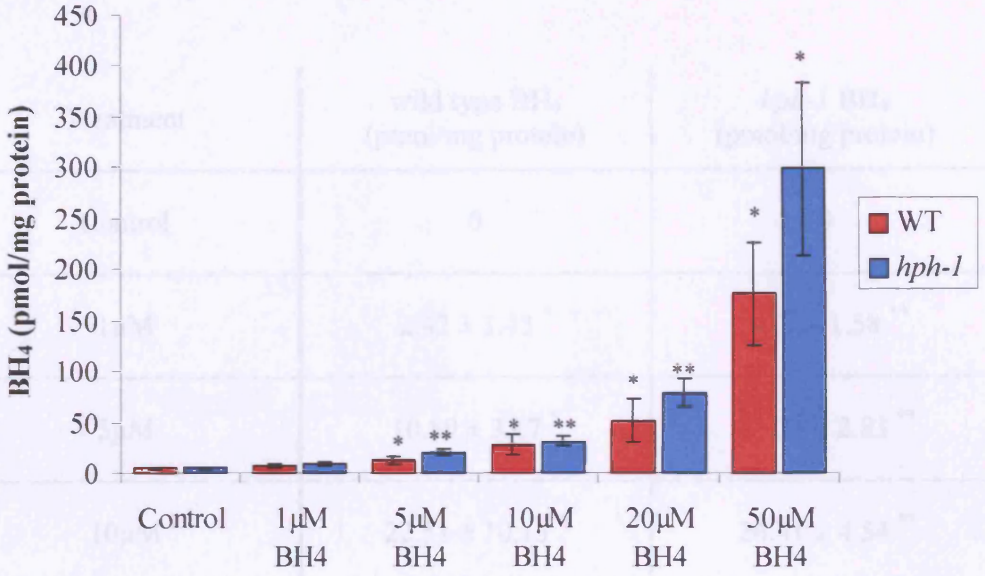


Figure 5.5. Effect of increasing exogenous concentrations of 5,6,7,8-tetrahydrobiopterin hydrochloride on intracellular BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes.

Astrocytes were treated for 3 hours with 1, 5, 10, 20 and 50μmol/litre BH_4. Following this treatment cell culture media was removed and cells were washed 3 times with 0.5ml Hank's Balanced Salt Solution (HBSS), and then harvested for BH_4 quantification. BH_4 was measured using the reverse-phase HPLC. Data is expressed as mean ± SEM, n = 3-7 of separate cell culture preparations. *, $p < 0.05$ vs. untreated control, **, $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test.

Treatment	wild type BH_4 (pmol/mg protein)	*hph-1* BH_4 (pmol/mg protein)
Control	0	0
1μM	2.42 ± 1.43 *	3.77 ± 1.58 **
5μM	10.89 ± 3.17 *	15.33 ± 2.83 **
10μM	22.51 ± 10.13 *	26.41 ± 4.54 **
20μM	46.51 ± 21.46 *	74.03 ± 13.73 **
50μM	170.75 ± 50.96 *	293.45 ± 84.31 **

Table 5.3. Effect of increasing exogenous concentrations of BH_4 on intracellular BH_4 levels in wild type (WT) and *hph-1* cortical astrocytes, minus basal levels.
Astrocytes were treated for 3 hours with 1, 5, 10, 20 and 50μmol/litre 5,6,7,8-tetrahydrobiopterin hydrochloride. Following this treatment cell culture media was removed and cells were washed 3 times with 0.5ml Hank's Balanced Salt Solution (HBSS), and then harvested for BH_4 quantification. BH_4 was measured using the reverse-phase HPLC. Data is expressed as mean ± SEM, n = 3-7 of separate cell culture preparations, minus basal values. *, $p < 0.05$ vs. untreated control, **, $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test.

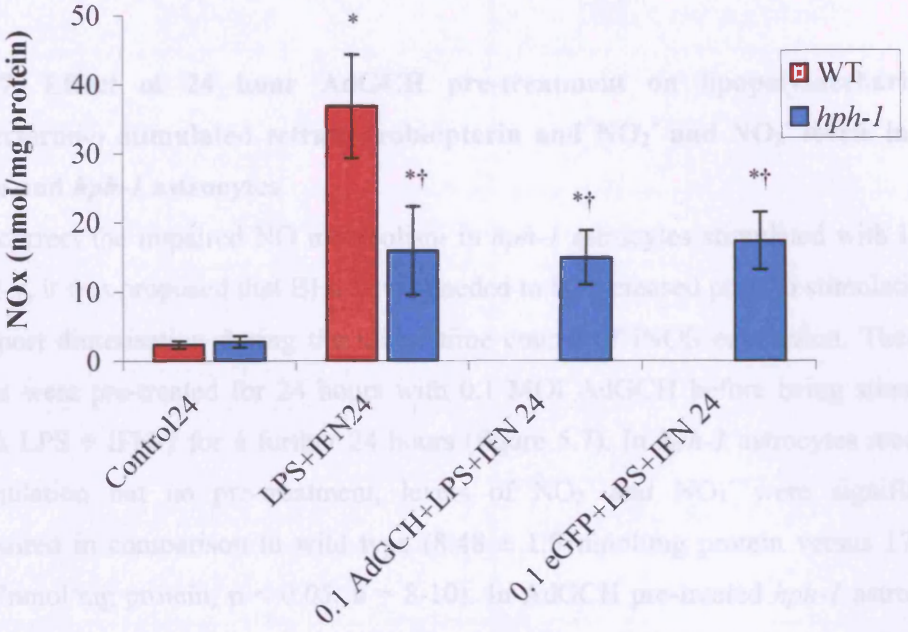


Figure 5.6. Effect of adenoviral transfection on NO_2^- and NO_3^- levels in cell culture media of wild type (WT) and *hph-1* astrocytes.

Astrocytes were incubated in the presence and absence of LPS + IFN-γ (1μg/ml and 100U/ml respectively), in combination with 0.1 multiplicity of infection units (MOI) AdGCH and 0.1 MOI AdeGFP. NO_2^- and NO_3^- was measured using the Griess assay. Data expressed as mean ± SEM, n = 3-9 of separate cell culture preparations. *, $p < 0.05$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. †, $p < 0.05$ vs. wild type LPS + IFN-γ treated, determined by one way ANOVA followed by least significant difference test.

augmented levels of BH_4 in these cells approximately 24-fold (table 5.4.) ($p > 0.05$, $n = 4$), although this was not significantly different from 0.1 MOI AdGCH treatment alone. AdeGFP in combination with LPS + IFN-γ did not raise BH_4 above LPS + IFN-γ stimulated levels.

5.4.7. Effect of 24 hour AdGCH pre-treatment on lipopolysaccharide + interferon-γ stimulated tetrahydrobiopterin and NO_2^- and NO_3^- levels in wild type and *hph-1* astrocytes

To correct the impaired NO metabolism in *hph-1* astrocytes stimulated with LPS + IFN-γ, it was proposed that BH_4 levels needed to be increased prior to stimulation, to support dimerisation during the initial time course of iNOS expression. Therefore cells were pre-treated for 24 hours with 0.1 MOI AdGCH before being stimulated with LPS + IFN-γ for a further 24 hours (figure 5.7). In *hph-1* astrocytes receiving stimulation but no pre-treatment, levels of NO_2^- and NO_3^- were significantly impaired in comparison to wild type (8.48 ± 1.57nmol/mg protein versus 17.62 ± 3.97nmol/mg protein; $p < 0.05$, $n = 8$-10). In AdGCH pre-treated *hph-1* astrocytes, NO_2^- and NO_3^- increased significantly to 14.81 ± 2.97nmoles/mg protein upon stimulation ($p < 0.05$, $n = 7$). Furthermore NO_2^- and NO_3^- levels following pre-treatment were no longer significantly different from wild type LPS + IFN-γ stimulated values, and AdeGFP had no effect (7.85 ± 2.16nmol/mg protein). Additionally, control levels in wild type and *hph-1* astrocytes, neither of which received pre-treatment or stimulation, were statistically identical, and 0.1 MOI AdGCH and AdeGFP had no effect on NO_2^- and NO_3^- alone (figure 5.7).

Corresponding BH_4 levels in wild type and *hph-1* astrocytes with these treatments (figure 5.8.) showed a similar pattern to the 24-hour co-treatments (table 5.4). Wild type basal levels (6.21 ± 1.07nmol/mg protein) remained significantly higher than *hph-1* (3.33 ± 0.48nmol/mg protein) ($p < 0.05$, $n = 5$-7), and LPS + IFN-γ stimulated *hph-1* astrocytes (10.29 ± 1.73nmol/mg protein) produced lower amounts of BH_4 than wild type (73.93 ± 25.47nmol/mg protein) ($p < 0.05$, $n = 5$-6). Treating *hph-1* astrocytes with 0.1 MOI AdGCH for 48-hours before stimulation significantly increased BH_4 above basal (384.16 ± 67.55nmol/mg protein) ($p < 0.01$, $n = 6$), and LPS + IFN-γ stimulated *hph-1* astrocytes ($p < 0.01$, $n = 6$), but also beyond 0.1 MOI AdGCH treated cells (248.21 ± 48.67nmol/mg protein) ($p < 0.05$, $n = 6$). The control

Treatment	BH_4 pmol/mg protein
WT Control	7.35 ± 1.28
hph-1 control	3.22 ± 0.36 †
WT LPS + IFN-γ 24	101.13 ± 31.22 *
hph-1 LPS + IFN-γ 24	6.31 ± 1.33 *†
hph-1 0.1 MOI AdGCH + LPS + IFN-γ 24	151.60 ± 29.86 †*
hph-1 0.1 MOI AdeGFP + LPS + IFN-γ 24	13.21 ± 7.11 *
hph-1 0.1 MOI AdGCH	197.39 ± 37.02 †*
hph-1 0.1 MOI AdeGFP	3.10 ± 0.60 *

Table 5.4. Effect of adenoviral pre-treatment plus LPS + IFN-γ stimulation on BH_4 levels in wild type (WT) and *hph-1* astrocytes.

Astrocytes were incubated in the presence and absence of LPS + IFN-γ (1μg/ml and 100U/ml respectively), in combination with 0.1 multiplicity of infection units (MOI) AdGCH or 0.1 MOI AdeGFP. Cells were harvested after 24 hours and BH_4 levels measured. Data expressed as mean ± SEM, n = 3-11 of separate cell culture preparations. †, $p < 0.05$ vs. wild type calculated by one way ANOVA followed by least significant difference test. *, $p < 0.05$ vs. untreated control as determined by one way ANOVA followed by least significant difference test.

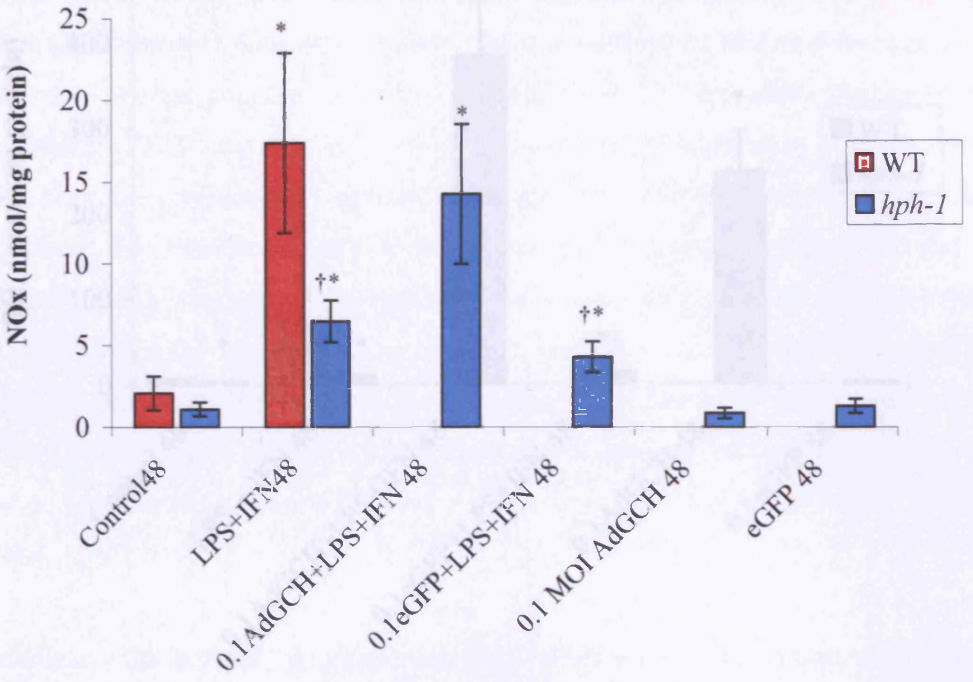


Figure 5.7. Effect of adenoviral pre-treatment on LPS + IFN-γ stimulated BH_4 levels in cell culture media of wild type (WT) and *hph-1* astrocytes.

Astrocytes were pre-treated for 24 hours in the presence or absence of 0.1 multiplicity of infection units (MOI) AdGCH or 0.1 MOI AdeGFP, then stimulated with LPS + IFN-γ (1μg/ml and 100U/ml respectively) for a further 24 hours. NO_2^- and NO_3^- was measured using the Griess assay. Data expressed as mean ± SEM, n = 4-7 of separate cell culture preparations. *, $p < 0.05$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. †, $p < 0.05$ vs. wild type LPS + IFN-γ stimulated as determined by one way ANOVA followed by least significant difference test.

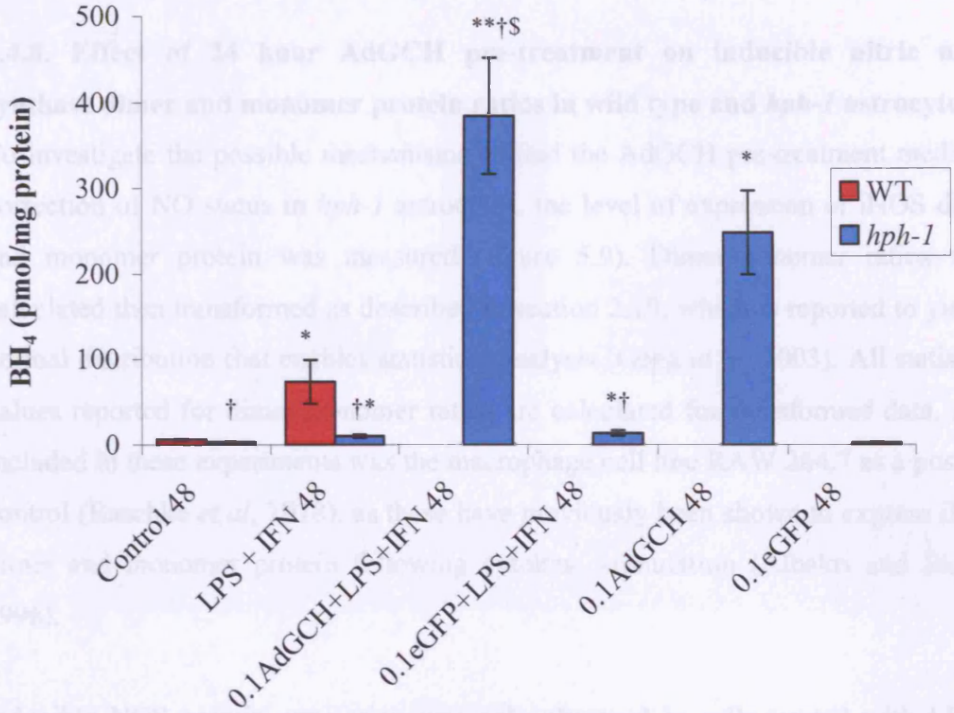


Figure 5.8. Effect of adenoviral pre-treatment on LPS + IFN-γ stimulated BH_4 levels in wild type (WT) and *hph-1* astrocytes.

Astrocytes were pre-treated for 24 hours in the presence or absence of 0.1 multiplicity of infection units (MOI) AdGCH or 0.1 MOI AdeGFP, then stimulated with LPS + IFN-γ (1μg/ml and 100U/ml respectively) for a further 24 hours. BH_4 was measured using the reverse-phase HPLC. Data expressed as mean ± SEM, n = 4-7 of separate cell culture preparations. *, $p < 0.05$ vs. untreated control, **, $p < 0.01$ vs. untreated control calculated using one way ANOVA followed by least significant difference test. †, $p < 0.05$ vs. wild type LPS + IFN-γ stimulated as determined by one way ANOVA followed by least significant difference test. $^{\$}$, $p < 0.05$ vs. 0.1 AdGCH treated cells calculated by one way ANOVA followed by least significant difference test.

virus AdeGFP in combination with LPS + IFN-γ stimulation did not increase BH_4 levels further than LPS + IFN-γ alone (13.99 ± 3.07nmol/mg protein) (figure 5.8).

5.4.8. Effect of 24 hour AdGCH pre-treatment on inducible nitric oxide synthase dimer and monomer protein ratios in wild type and *hph-1* astrocytes

To investigate the possible mechanisms behind the AdGCH pre-treatment mediated correction of NO status in *hph-1* astrocytes, the level of expression of iNOS dimer and monomer protein was measured (figure 5.9). Dimer:monomer ratios were calculated then transformed as described in section 2.10, which is reported to yield a normal distribution that enables statistical analysis (Gegg *et al*, 2003). All statistical values reported for dimer:monomer ratios are calculated for transformed data. Also included in these experiments was the macrophage cell line RAW 264.7 as a positive control (Raschke *et al*, 1978), as these have previously been shown to express iNOS dimer and monomer protein following cytokine stimulation (Albakri and Stuehr, 1996).

Inducible NOS protein expression was only observed in cells treated with LPS + IFN-γ in these experiments, including RAW 264.7 macrophage cell lines. Analysis of dimer:monomer ratios revealed approximately 6-fold greater levels of dimerisation in wild type astrocytes (0.461 ± 0.05) compared to *hph-1* (0.073 ± 0.02)($p < 0.01$, n = 3-4). When *hph-1* astrocytes were pre-treated for 24 hours with 0.1 MOI AdGCH the ratio of dimer:monomer was significantly augmented from 0.073 ± 0.02 to 0.365 ± 0.08 ($p < 0.05$, n = 4), such that differences from the wild type were no longer apparent. AdeGFP pre-treatment appeared to have no influence on levels of dimerisation.

5.4.9. Effect of 24 hour AdGCH pre-treatment on inducible nitric oxide synthase total protein levels in wild type and *hph-1* astrocytes

In addition to the ratio of dimer to monomer, the total amount of iNOS protein was quantified in LPS + IFN-γ stimulated wild type and *hph-1* astrocytes. Total iNOS protein was calculated by measuring the intensity of bands at 130kDa. Boiling samples removed 260kDa bands, which has been previously documented (Ohtsuka *et al*, 2002; Ravi *et al*, 2004), and further confirms the identity of the bands.

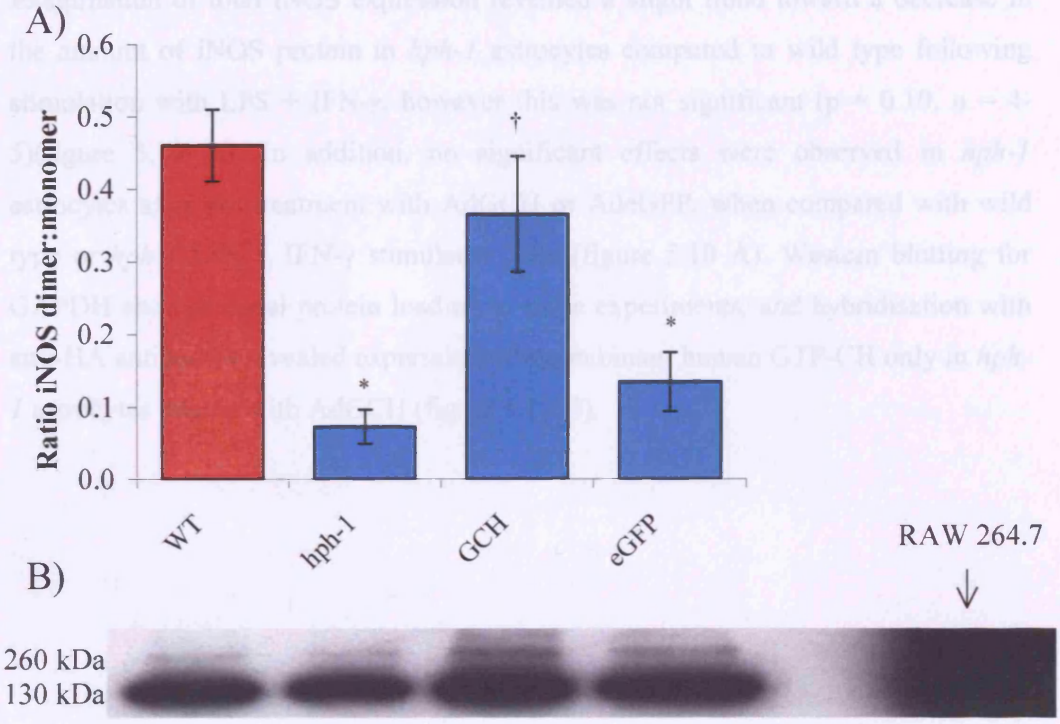


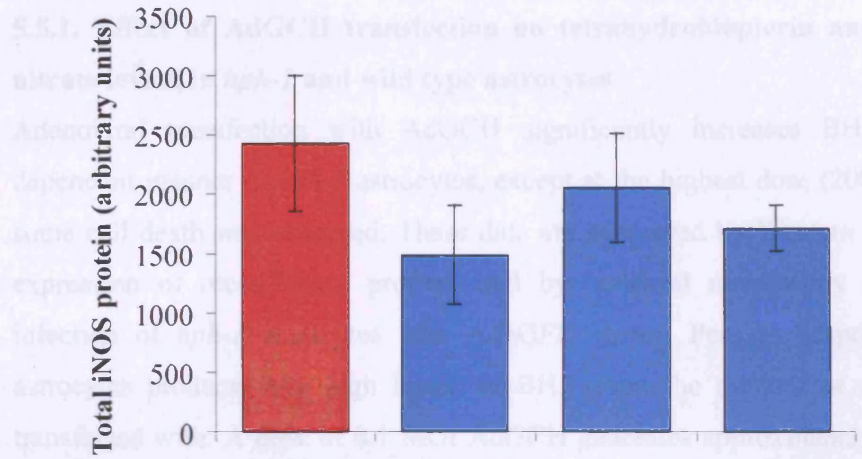
Figure 5.9. Effect of adenoviral pre-treatment on iNOS dimer:monomer ratio in LPS + IFN-γ stimulated wild type (WT) and *hph-1* astrocytes.

A) Astrocytes and RAW 264.7 cell lines were pre-treated for 24 hours in the presence or absence of 0.1 multiplicity of infection units (MOI) AdGCH or 0.1 MOI AdeGFP, then stimulated with LPS + IFN-γ (1μg/ml and 100U/ml respectively) for a further 24 hours. Western blotting was carried out as described in section 2.7. Cell lysates were fractionated by SDS–PAGE and immunoblotted with antibodies to iNOS. Bands were quantified using GelPro analysis system and software (Media Cybernetics). Data expressed as mean ± SEM, n = 3-4 separate cell culture preparations. *, $p < 0.05$ vs. wild type LPS + IFN-γ stimulated cells calculated using one way ANOVA followed by least significant difference test. †, $p < 0.05$ vs. *hph-1* LPS + IFN-γ stimulated as determined by one way ANOVA followed by least significant difference test.

B) Western blot showing presence of iNOS dimer and monomer in wild type and *hph-1* astrocytes and RAW 264.7 cell lines following LPS + IFN-γ stimulation.

Examination of total iNOS expression revealed a slight trend toward a decrease in the amount of iNOS protein in *hph-1* astrocytes compared to wild type following stimulation with LPS + IFN-γ, however this was not significant ($p = 0.10$, $n = 4$-5)(figure 5.10 A). In addition, no significant effects were observed in *hph-1* astrocytes after pre-treatment with AdGCH or AdeGFP, when compared with wild type or *hph-1* LPS + IFN-γ stimulated cells (figure 5.10 A). Western blotting for GAPDH showed equal protein loading in these experiments, and hybridisation with anti-HA antibodies revealed expression of recombinant human GTP-CH only in *hph-1* astrocytes treated with AdGCH (figure 5.10 B).

A)



B)

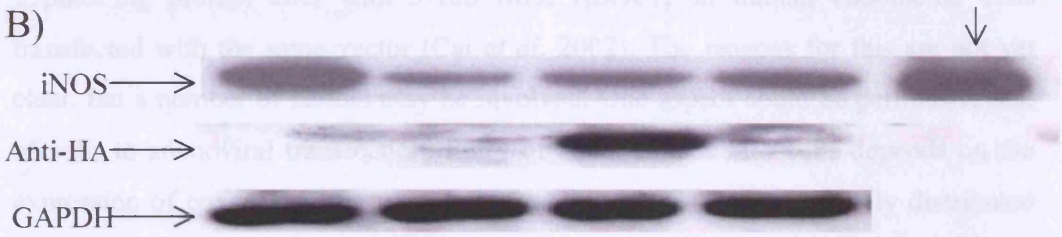


Figure 5.10. Effect of adenoviral pre-treatment on total iNOS protein in LPS + IFN-γ stimulated wild type and *hph-1* astrocytes.

A) Astrocytes and RAW 264.7 cell lines were pre-treated for 24 hours in the presence or absence of 0.1 multiplicity of infection units (MOI) AdGCH or 0.1 MOI AdeGFP, then stimulated with LPS + IFN-γ (1μg/ml and 100U/ml respectively) for a further 24 hours. Data expressed as mean ± SEM, n = 4-5 separate cell culture preparations. Western blotting was carried out as described in section 2.7. Cell lysates were fractionated by SDS–PAGE and immunoblotted with antibodies to HA tag epitope, GAPDH and iNOS. Bands were quantified using GelPro analysis system and software (Media Cybernetics) and the density measured in arbitrary units.

B) Representative Western blots of iNOS, recombinant human GTP-CH from AdGCH and GAPDH.

5.5. DISCUSSION

5.5.1. Effect of AdGCH transfection on tetrahydrobiopterin and nitrite and nitrate levels, in *hph-1* and wild type astrocytes

Adenoviral transfection with AdGCH significantly increases BH_4 in a dose-dependent manner in *hph-1* astrocytes, except at the highest dose (200 MOI) where some cell death was observed. These data are supported by Western blots showing expression of recombinant protein, and by confocal microscopy demonstrating infection of *hph-1* astrocytes with AdeGFP vector. Perhaps surprisingly, *hph-1* astrocytes produce very high levels of BH_4 given the amount of virus they are transfected with. A dose of 0.1 MOI AdGCH generates approximately 200pmol/mg protein BH_4, which is considerably higher than previously reported levels of 25-75pmol/mg protein BH_4 with 5-100 MOI AdGCH in human endothelial cells transfected with the same vector (Cai *et al*, 2002). The reasons for this are not yet clear, but a number of factors may be involved. One aspect could be permissiveness of cells to adenoviral transfection. Entry of adenoviruses into cells depends on the expression of coxsackievirus and adenovirus receptors (CAR), a widely distributed type 1 membrane protein (Roelvink *et al*, 1998). Susceptibility of cells to adenoviral mediated gene transfer is directly correlated to the level of CAR expression (Miller *et al*, 1998). Astrocytes highly express CAR, with more than 50% of cells expressing the receptor (Fueyo *et al*, 2003). In contrast, endothelial cells are transduced by both viral and non-viral vectors with low efficiency compared to other cell types (Nicklin *et al*, 2004). Therefore this may be why gene transfer with AdGCH generates more BH_4 production in *hph-1* astrocytes than human endothelial cells.

A study investigating the toxicity of anticancer drugs shows adenoviruses can initiate functional effects in astrocytes at just 0.1 MOI viral titre (Maron *et al*, 1997). This suggests that the results obtained with such a low amount of AdGCH in *hph-1* astrocytes are not necessarily unique, and that adenoviral transfection may be extremely efficient in this cell type. However, another potential explanation for the high concentrations of BH_4 found in *hph-1* astrocytes, could be species differences in the forms of GTP-CH and GFRP found in human and mouse cells. GTP-CH activity is highly regulated by the feedback regulatory protein GFRP, which responds to BH_4 and phenylalanine by inhibiting and stimulating GTP-CH activity, respectively

(Harada *et al*, 1993; Milstien *et al*, 1996). The human form of GTP-CH is used to transfect mouse cells in these experiments. Therefore, differences in the amino acid sequence of human GTP-CH could mean that the mouse form of GFRP is unable to inhibit the human form of GTP-CH, resulting in uncontrolled activity of transfected GTP-CH and very high concentrations of BH_4. Human and mouse GFRP differ by three amino acids, enough difference to make antiserum specific for human GFRP unreactive for mouse GFRP (Professor G. Werner-Felmayer, Innsbruck Medical University, Innsbruck, Austria, personal communication).

However, studies in GTP-CH transgenic mice (*GCH*-Tg), a mouse line with endothelial-targeted over-expression of the human form of GTP-CH, suggests that mouse form of GFRP can inhibit human GTP-CH (Alp *et al*, 2003; Meininger *et al*, 2004). In *GCH*-Tg mice, expression of recombinant human GTP-CH is found in tissues rich with endothelial cells, such as lung, liver, and aorta. In these tissues, although the total amount of GTP-CH is elevated 5-10 fold, BH_4 levels only rise up to 3-fold (Alp *et al*, 2003). If mouse GFRP cannot regulate human GTP-CH, it would be expected that very high levels of BH_4 would be generated in the tissues of these animals; however this is not the case in *GCH*-Tg mice.

Another possible reason for high concentrations of BH_4 in *hph-1* astrocytes following gene transfer, is that multiple translations of the recombinant human *GCH* transcript results in high quantities of GTP-CH protein in each cell. A way to investigate this would be to use *in situ* hybridisation to compare expression of recombinant with native forms of the enzyme. This may give an idea of how much enzyme is expressed from AdGCH. A further possibility is that transfected human GTP-CH somehow influences expression of the native form; however in *GCH*-Tg mice human GTP-CH had no impact on the quantity of native enzyme detected (Alp *et al*, 2003).

Although BH_4 levels increase following AdGCH gene transfer, basal NO_2^- and NO_3^- remain unaffected in *hph-1* astrocytes. Furthermore, *hph-1* cells do not respond to sepiapterin, suggesting that under basal conditions BH_4 is not limiting for NO metabolism in these cells (see also section 4.4.4). The specific activity of NOS in *hph-1* astrocytes is undetectable in non-stimulated cells (Barker *et al*, 1998). In addition, iNOS expression is not detectable in *hph-1* astrocytes under basal

conditions in our experiments, or in previous studies (Barker *et al*, 1998). Therefore the primary source of NO is likely to be nNOS or eNOS. Both nNOS and eNOS produce picomolar amounts of NO, whereas iNOS is capable of micromolar quantities (Geller and Billiar, 1998). Since the limits of detection of NO_2^- and NO_3^- are around 1 nanomolar for this assay, it is unlikely that increased BH_4 would result in accumulation of NO in *hph-1* astrocytes. Studies in murine CAD cells, an nNOS-expressing neuronal line, show that 50μM sepiapterin applied to unstimulated cells did not cause nitrite levels to rise (Xu *et al*, 2004). Therefore, simply increasing BH_4 levels is not enough to show correction of NO metabolism in *hph-1* astrocytes.

5.5.2. Uptake of tetrahydrobiopterin into *hph-1* and wild type astrocytes

Due to the relatively high concentrations of BH_4 generated following AdGCH transfection in *hph-1* astrocytes, the uptake BH_4 into these cells was investigated, and BH_4 accumulation compared with wild type astrocytes. In theory at 0.1 MOI, only 1 in 10 *hph-1* astrocytes are transfected with AdGCH, which elevates concentrations of BH_4 50-fold. These experiments were carried out to observe whether BH_4 can be taken up by surrounding cells, and if impaired BH_4 metabolism has an impact on cofactor accumulation. As the amount of BH_4 applied to cells increases, intracellular levels of BH_4 rise dose-dependently in wild type and *hph-1* astrocytes. When the uptake of BH_4 in wild type and *hph-1* is compared, no differences are found between these cells. These data show that astrocytes can take up BH_4, and that the small proportion of cells transfected by AdGCH may be able to produce BH_4 that neighbouring cells can utilise. This raises the possibility that for *in vivo* work only a small proportion of astrocytes may need to receive adenovirus to produce BH_4 for the rest of the population.

BH_4 release and uptake has been studied *in vivo* (Sawabe *et al*, 2004), as well as in human endothelial cells, hepatocytes, PC12, rat synaptosomes and rat striatal tissue (Anastasiadis *et al*, 1996; Cai *et al*, 2002; Sawabe *et al*, 2005). Depending on the cell type BH_4 is taken up directly via passive diffusion that is not glucose or sodium dependent, or by a methotrexate sensitive indirect pathway (Anastasiadis *et al*, 1996; Sawabe *et al*, 2005). It is proposed in the indirect pathway BH_4 is first oxidised to BH_2, then converted back to BH_4 inside the cell via DHFR (Sawabe *et al*, 2004, 2005). It is not clear which of the pathways is in operation in these cells, however

DHFR expression (Kaufman, 1991) and activity (Ludwig *et al*, 1987) are low in the brain compared to other organs. Therefore it is more likely that the direct pathway is in operation in *hph-1* and wild type astrocytes. Additional experiments with methotrexate, an inhibitor of DHFR would be necessary to explore this further.

5.5.3. Effect of AdGCH treatment on lipopolysaccharide + interferon-γ stimulated tetrahydrobiopterin and nitrite and nitrate levels in wild type and *hph-1* astrocytes

Stimulating *hph-1* and wild type astrocytes with LPS + IFN-γ increases NO_2^- and NO_3^- levels in both cell types, however in *hph-1* cells this response is attenuated by approximately 50%. Results from section 4.4.5. demonstrate that increasing BH_4 via sepiapterin can abolish differences between wild type and *hph-1*, so AdGCH was used to attempt to increase NO. However, when cells were treated with AdGCH at the same time point as LPS + IFN-γ, after 24 hours although BH_4 levels increased, *hph-1* NO_2^- and NO_3^- did not change. A time course of iNOS and GTP-CH expression reveals these enzymes are induced in parallel following LPS + IFN-γ (Hattori and Gross, 1993). Sepiapterin potentiates NO synthesis in LPS + IFN-γ stimulated rat aortic smooth muscle cells, and shifts the profile of induction to earlier time points (Gross and Levi, 1992). If BH_4 is increased at the same time as iNOS induction, production of NO is shifted to earlier times and rises at a steeper rate (Gross and Levi, 1992). Therefore, BH_4 may limit the onset and magnitude of the NO response. It is possible there is a delay in the production of BH_4 from AdGCH, from the vector being transported across the cell membrane and into the nucleus, then from the time taken to express GTP-CH from AdGCH. Delayed BH_4 production from AdGCH may result in diminished availability of BH_4 to promote iNOS dimerisation. This delay may explain why AdGCH does not potentiate NO_2^- and NO_3^- production in LPS + IFN-γ stimulated *hph-1* astrocytes, when transfection occurs at the same time as stimulation.

5.5.4. Effect of 24 hour AdGCH pre-treatment on lipopolysaccharide + interferon-γ stimulated tetrahydrobiopterin and nitrite and nitrate levels in wild type and *hph-1* astrocytes

To augment NO production in LPS + IFN-γ stimulated *hph-1* astrocytes to wild type levels, cells were pre-treated with AdGCH for 24 hours to allow BH_4 to increase

sufficiently to support NO synthesis. NO_2^- and NO_3^- are significantly elevated in *hph-1* cells treated this way, and are statistically identical to wild type. BH_4 is known to have a number of roles in NO synthesis. It increases the affinity of NOS for arginine leading to greater activity of this enzyme (Brand *et al*, 1995; Klatt *et al*, 1994). Inducible NOS is only active as a homodimer (Ghosh *et al*, 1995), and BH_4 is required for both dimer formation and to stabilise the enzyme in this structure (Baek *et al*, 1993; Tzeng *et al*, 1995). Furthermore, BH_4 promotes uptake of arginine into cells (Schwartz *et al*, 2001). The combination of these factors may explain why impairment of BH_4 metabolism results in attenuated LPS + IFN-γ stimulated NO synthesis in *hph-1* astrocytes, and why increasing BH_4 via AdGCH corrects NO generation. The contribution of these mechanisms is scrutinized in further detail in section 4.5.2. Furthermore, the role of BH_4 in iNOS dimerisation and the total amount of iNOS protein is examined below in sections 5.5.5. and 5.5.6.

hph-1 cells pre-treated with 0.1 MOI AdGCH then stimulated with LPS + IFN-γ generated more BH_4 than 0.1 MOI AdGCH alone. The amount of BH_4 produced appears to be greater than the sum of transfection and stimulation. These data contrast with section 4.4.5., where sepiapterin combined with LPS + IFN-γ did not improve BH_4 beyond the individual treatments. Other literature describes the influence of sepiapterin in combination with LPS + IFN-γ on NO stimulation, but these studies do not report BH_4 levels (Gross and Levi, 1992; Nakayama *et al*, 1994). One reason for these results may be that LPS + IFN-γ increases activity of transfected GTP-CH. LPS + IFN-γ stimulates activity of GTP-CH, but not the other enzymes in the biosynthetic pathway, including sepiapterin reductase (Werner-Felmayer *et al*, 1993). Furthermore, NO can suppress GFRP expression, thus enhancing GTP-CH activity (Park *et al*, 2002). Therefore if there are any inhibitory effects of *hph-1* GFRP on human GTP-CH from AdGCH, the increase in NO that was observed may suppress the action of GFRP leading to greater BH_4 production. However, further work is necessary to investigate these results.

5.5.5. Effect of 24 hour AdGCH pre-treatment on inducible nitric oxide synthase dimer and monomer protein ratios in wild type and *hph-1* astrocytes

Homodimerisation is essential for iNOS activity (Baek *et al*, 1993). Factors required for dimer formation include calmodulin, FAD, FMN, NADPH and haem (Baek *et al*,

1993). For dimers to become stable and active enzyme structures, BH_4 and arginine binding are essential (Baek *et al*, 1993; Ghosh *et al*, 1997). It was proposed that the impaired NO synthesis observed in LPS + IFN-γ stimulated *hph-1* astrocytes was the result of reduced iNOS dimerisation arising from impaired BH_4 production. To investigate this, iNOS dimer and monomer protein were compared using Western blotting (Klatt *et al*, 1995). When the ratio of iNOS dimer:monomer is calculated in stimulated *hph-1* and wild type astrocytes, it is found that *hph-1* had significantly less dimer complex than wild type.

These data suggest that BH_4 availability impacts on the dimerisation of iNOS enzyme in LPS + IFN-γ stimulated cells. So far a number of studies have looked at the influence of different factors on iNOS dimerisation. Most are under dictated experimental conditions using recombinant protein or partial domains, rather than in cells or tissues. However, there is some literature especially relevant to the results presented here. Experiments using phosphoinositide 3-kinase (PI3K) deficient macrophages investigated the relationship between BH_4, NO, GTP-CH expression, iNOS dimerisation and total iNOS following LPS + IFN-γ stimulation (Sakai *et al*, 2006). PI3K deficient macrophages display lower concentrations of BH_4, along with attenuated expression of *GCH* mRNA and GTP-CH protein after stimulation (Sakai *et al*, 2006). This is coupled with decreased NO, and impaired iNOS dimerisation in comparison to wild type (Sakai *et al*, 2006). Ratios of dimer:monomer compare favourably between data presented here and in Sakai *et al*, 2006, which provides additional evidence that the bands detected were iNOS. In wild type cells a ratio of 0.45 and 0.4 were reported in this thesis and in Sakai *et al*, 2006, respectively. In PI3K mutant macrophages the ratio was 0.2 (Sakai *et al*, 2006), whereas *hph-1* astrocytes values were approximately 0.07. This may reflect the degree of BH_4 deficiency. BH_4 levels in *hph-1* astrocytes were around 14% of wild type after LPS + IFN-γ stimulation, whereas the relative deficiency of BH_4 in PI3K cells is 50%.

When *hph-1* astrocytes were pre-treated with AdGCH for 24 hours, an elevation in the level of dimerisation is observed following LPS + IFN-γ stimulation. The amount of dimer present is increased to within the wild type range, demonstrating that the amount of BH_4 is associated with the ability of cells to produce active iNOS dimers. When BH_4-deficient PI3K cells were incubated with L-sepiapterin during LPS +

IFN-γ stimulation, levels of dimer found in the cells were also increased to within wild type range (Sakai *et al*, 2006). An enhancement of iNOS dimerisation may also be why NO metabolism can be increased after L-sepiapterin treatment in *hph-1* LPS + IFN-γ stimulated astrocytes in section 4.4.5. This suggests that the level of BH_4 is critical to iNOS dimerisation, and that gene transfer with AdGCH may be a viable approach to correct NO synthesis in the *hph-1* mouse.

Although BH_4 is found in abundance in wild type and AdGCH transfected *hph-1* astrocytes, most of the iNOS found in the cell was still present as monomer. In wild type and AdGCH plus LPS + IFN-γ stimulated *hph-1* cells, approximately 30% of protein was dimer, in RAW 264.7 cells used as a positive control this was lower at 20% (data not shown), and in *hph-1* astrocytes without AdGCH this figure was 10%. These data correspond well with levels in macrophages (Sakai *et al*, 2006) and RAW 264.7 cells (Albakri and Stuehr, 1996), where in both cases the majority of iNOS was monomeric. Of the NOS subtypes, iNOS is the least stable isoform, followed by nNOS, with eNOS being the most durable (Panda *et al*, 2002). In both the presence and absence of arginine or BH_4, iNOS is the most easily dissociated, either by temperature or chemical disruption (Panda *et al*, 2002). Reports suggest the dissociation of iNOS may be mediated by NO (Chen *et al*, 2002; Albakri and Stuehr, 1996). In RAW 264.7 cells iNOS accumulates in a linear manner over 16 hours (Albakri and Stuehr, 1996). Dimer also increases, but at a slower rate, and this slowly tails off until approximately 25% of iNOS is dimer. When iNOS activity is inhibited using N^{ω}-nitro-L-arginine methyl ester (L-NAME), formation of dimer increases, suggesting that NO synthesis blocks dimer assembly. However, L-NAME also reduces the total amount of iNOS protein by half (Albakri and Stuehr, 1996).

It is believed NO prevents dimer assembly either by limiting haem insertion into the enzyme (Albakri and Stuehr, 1996; Chen *et al*, 2002), or by S-nitrosation of the zinc tetrathiolate cysteine in iNOS, resulting in release of zinc from the enzyme and loss of dimer (Mitchell *et al*, 2005). However, the issue of NO preventing iNOS dimerisation is not straightforward. Some groups report that NO disrupts dimer assembly (Albakri and Stuehr, 1996; Chen *et al*, 2002; Li *et al*, 2006), whilst others claim it promotes dimer formation (Park *et al*, 2002). When rat hepatocytes were pre-treated with S-Nitroso-N-acetyl-*dl*-penicillamine (SNAP), an NO donating molecule,

BH_4 and GTP-CH, and NO production and dimerisation all increased after IL-1β and IFN-γ stimulation (Park *et al*, 2002). However, the same experiment in two different cell types, including RAW 264.7 cells, had no effect on dimerisation or NO levels. Therefore these effects may be cell specific (Park *et al*, 2002). In *hph-1* astrocytes it is not clear if NO produced from iNOS reduces the proportion of dimer over time. However, there is an association between the amount of BH_4 present and iNOS dimerisation, which results in greater NO production. Treating *hph-1* astrocytes with AdGCH can correct the deficiency in NO metabolism, by providing more BH_4 for iNOS dimer assembly.

5.5.6. Effect of 24 hour AdGCH pre-treatment on inducible nitric oxide synthase total protein levels in wild type and *hph-1* astrocytes

Analysis of the total amount of iNOS protein in astrocytes stimulated with LPS + IFN-γ, did not reveal differences in expression between *hph-1* and wild type, either in the presence or absence of AdGCH or AdeGFP. There was slight trend toward decreased iNOS in *hph-1* cells in comparison to wild type, however this did not reach significance ($p = 0.10$, $n = 4\text{-}5$). Previous studies looking at the amount of iNOS protein following stimulation demonstrate increased total iNOS expression and activity in *hph-1* astrocytes, when compared to wild type (Barker *et al*, 1998). It is suggested greater expression and activity arises from NO limiting NFκB-dependent iNOS expression (Park *et al*, 1997); thus lower production of NO in *hph-1* cells leads to augmented iNOS protein and activity (Barker *et al*, 1998). These results were obtained using enzyme linked immunosorbant assay (ELISA). It is possible this assay is more sensitive changes in protein levels, so perhaps if the number of samples was increased for these experiments similar results may be obtained.

In macrophages from the PI3K mouse line, BH_4 and NO levels, GTP-CH expression and iNOS dimerisation are all shown to be reduced upon LPS + IFN-γ stimulation in comparison to wild type (Sakai *et al*, 2006). However, iNOS protein levels remain unaffected. In rat hepatocytes, addition of BH_4 to iNOS stimulated cells does not influence total iNOS expression (Loughran *et al*, 2005), and pre-treating these cells with an NO-donor also has no effect (Park *et al*, 2002). But when NO synthesis in LPS + IFN-γ stimulated RAW 264.7 cells was blocked, although the proportion of iNOS dimer increased, total levels of protein were halved (Albakri and Stuehr,

1996).Taken together these data provide contrasting viewpoints on whether changes in the levels of BH_4 or NO have an influence on total iNOS protein. The work from this chapter in *hph-1* astrocytes would suggest that a BH_4 deficiency has no influence on iNOS protein, but perhaps if iNOS protein was measured by two different assays such as Western blotting and ELISA, a clearer picture may be formed.

5.6. CONCLUSIONS

The deficiency of BH_4 and also NO metabolism in *hph-1* astrocytes can be corrected using adenovirus-mediated gene transfer of GTP-CH. AdGCH increases BH_4 dose-dependently in *hph-1* cells, with one virus particle per ten cells leading to an approximate 50-fold up-regulation of BH_4 content in cells. Stimulation of *hph-1* astrocytes with LPS + IFN-γ increases iNOS expression and NO synthesis, however the level of NO generation and iNOS dimerisation is impaired when compared to wild type. Simultaneous treatment of AdGCH plus LPS + IFN-γ in *hph-1* astrocytes did not augment NO levels, however 24-hour AdGCH pre-treatment yields higher NO generation, as well as augmented iNOS dimer formation that was no longer lower than wild type. Analysis of the total iNOS protein demonstrated no significant differences between wild type or *hph-1*, both in the presence and absence of AdGCH.

These results are encouraging for the future *in vivo* work using AdGCH in the *hph-1* mouse, as the level of BH_4 obtained in *hph-1* cells with just 0.1 MOI AdGCH may make this curative approach very efficient. If the adenovirus is administered to these animals, transfecting a small proportion of cells may produce enough BH_4 to increase BH_4 in neighbouring cells, and correct the impaired BH_4, NO and monoamine deficiencies in these mice.

Chapter 6

General discussion and future work

6.1. GENERAL DISCUSSION

Tetrahydrobiopterin (BH_4) is an essential cofactor for TH, TRH, and NOS in the brain. Thus, it has a vital role in brain cathecholamine, 5-HT and NO metabolism. BH_4 is synthesized from GTP via three enzymatic stages, the first of which is the initial rate-limiting step, catalysed by GTP cyclohydrolase 1 (GTP-CH). Inborn errors of BH_4 metabolism arise from mutations to the genes involved in BH_4 metabolism, and a number of conditions such as PD (Lovenberg *et al*, 1979; Williams *et al*, 1980) and AD (Barford *et al*, 1984; Casal *et al*, 2003) have associated acquired BH_4 deficiencies. Autosomal recessive mutations to the gene encoding GTP-CH result in a debilitating syndrome where sufferers synthesize virtually no BH_4 and experience profound neurological symptoms, including severe mental retardation in the absence of treatment (Blau *et al*, 2001). The autosomal dominant form of GTP-CH deficiency is known as dopa-responsive dystonia (DRD), which manifests itself in a partial BH_4 deficiency, and is less severe but is accompanied by neurological symptoms if left untreated (Segawa *et al*, 2003).

An animal model of BH_4 deficiency has been developed, known as the *hph-1* mouse, which has a partial deficiency of BH_4 arising from defective GTP-CH (Gütlich *et al*, 1994). This mouse has impaired DA, 5-HT and NO metabolism, and is therefore proposed as a model for BH_4 deficiency (Hyland *et al*, 1996, 2003). The aim of this thesis was to further investigate the biochemistry of the *hph-1* mouse, both in whole brain tissue, as well as in astrocytes derived from the *hph-1*, then use gene therapy to correct the defective BH_4 and NO metabolism found in the mouse, by upregulating GTP-CH expression. This is with an ultimate view to treating patients with inborn errors of BH_4 metabolism using gene therapy.

Initial background work further demonstrated that BH_4 metabolism is impaired in the brain of the *hph-1* mouse. Levels of BH_4 in the brain were approximately 30% of wild type. Furthermore, the ability of the *hph-1* biosynthetic pathway to produce BH_4 from the initial substrate GTP was reduced. Residual activity of this pathway combined with levels of BH_4 in the brain further support evidence of a partial GTP-CH deficiency in the *hph-1* mouse (Gütlich *et al*, 1994; Barker *et al*, 1998). Activity of the BH_4 recycling pathway remains unaffected in the *hph-1* brain, which provides

assurance that if gene therapy was used to increase GTP-CH expression, an intact recycling system exists to maintain BH_4 levels. When "DHNTP" was introduced downstream of the metabolic block, levels of BH_4 comparable with wild type were synthesized in brain cytosol. This indicates that the other enzymes in the BH_4 biosynthetic pathway function normally, and further suggests that GTP-CH is the site of the *hph-1* metabolic block. In addition, the BH_4 deficiency in the *hph-1* mouse impacts on the levels of monoamine neurotransmitters and their metabolites, as NA, DA and its metabolite HVA, as well as 5-HIAA, the metabolite of 5-HT, were all found to be lower in the *hph-1* brain.

Experiments using cultured cortical *hph-1* astrocytes provided more evidence of impaired BH_4 and NO status. Basal levels of BH_4 were lower in *hph-1* cells, however NO_2^- and NO_3^- were comparable to wild type, suggesting that BH_4 may not be limiting for NOS under basal conditions. Stimulation with LPS + IFN-γ, elevated BH_4 and NO_2^- and NO_3^- in wild type and *hph-1* cells, however in *hph-1* astrocytes the BH_4 and NO response were significantly impaired. *hph-1* astrocytes also generate less BH_4 following L-sepiapterin treatment. This may suggest that the impairment of GTP-CH in these cells reduces either sepiapterin reductase or DHFR activity, although further work must verify this. When *hph-1* astrocytes were stimulated with LPS + IFN-γ, and had BH_4 levels augmented via L-sepiapterin, levels of NO_2^- and NO_3^- rose to within wild type range. These data suggest that transfecting *hph-1* astrocytes with an adenoviral vector containing GTP-CH could be an approach for correcting both the BH_4 and NO status of these cells.

Deficiency of BH_4 and also NO metabolism in *hph-1* astrocytes can be corrected using the adenovirus AdGCH. AdGCH contains the gene for recombinant human GTP-CH (Cai *et al*, 2002), and increases BH_4 dose-dependently in *hph-1* cells, with one virus particle per ten cells leading to approximately 50-fold up-regulation of BH_4. Efficiency of the viral transfer is confirmed by Western blotting showing expression of recombinant GTP-CH protein, and by fluorescence microscopy demonstrating expression of viral proteins within *hph-1* astrocytes. The reason for the high efficiency of uptake and expression in *hph-1* astrocytes is not presently clear, although literature suggests that this effect may not be unique (Maron *et al*, 1997). Astrocytes express a high concentration of CAR receptors needed to mediate

transport of virus into the cell (Fueyo *et al*, 2003). Thus, a high proportion of viral particles are likely to be taken up by *hph-1* cells. This may have useful implications for future work *in vivo* in the *hph-1* mouse, as a lower viral titre could be used to produce functional effects if administered to the brain. Furthermore, BH_4 produced from the AdGCH transfected astrocytes may be taken up by neighbouring cells (Sawabe *et al*, 2005), demonstrating that transfected cells may act as a "sink", generating BH_4 that can be utilised throughout the organism.

Simultaneous treatment of AdGCH plus LPS + IFN-γ in *hph-1* astrocytes did not augment extracellular NO_2^- and NO_3^-, however 24-hour pre-treatment with virus yielded NO generation that was comparable to wild type. This may be the result of a difference between the time course of iNOS stimulation and the time taken for AdGCH to infect *hph-1* astrocytes then express GTP-CH. When the molecular mechanisms behind the increase in NO_2^- and NO_3^- were investigated, it was discovered that *hph-1* astrocytes had a lower proportion of iNOS dimer to monomer in comparison to wild type. When cells were pre-treated with AdGCH for 24 hours, the proportion of the dimer increased. However, the amount of iNOS protein present in the cells was not different between *hph-1* and wild type, and did not alter following AdGCH pre-treatment. These results of AdGCH viral transfection in *hph-1* astrocytes are encouraging for the future *in vivo* work in the mouse, as the level of BH_4 obtained in *hph-1* cells with just 0.1 MOI AdGCH may make this curative approach very efficient. In addition the molecular mechanisms behind the observed impairment of NO synthesis in *hph-1* astrocytes appear to have been elucidated, i.e. the impaired ability of *hph-1* astrocytes to produce iNOS dimers. A summary illustration of the key findings in *hph-1* astrocytes is shown in figure 6.1.

The results from the experiments in this thesis have revealed additional information about the biochemical characteristics of the *hph-1* mouse, and have demonstrated the potential of gene therapy in correcting the metabolic defect. These data may eventually be useful as a treatment strategy for sufferers of inborn errors of BH_4 metabolism, and offer a more long-term approach to alleviating the associated impairment of phenylalanine, monoamine neurotransmitter and NO metabolism in these individuals.

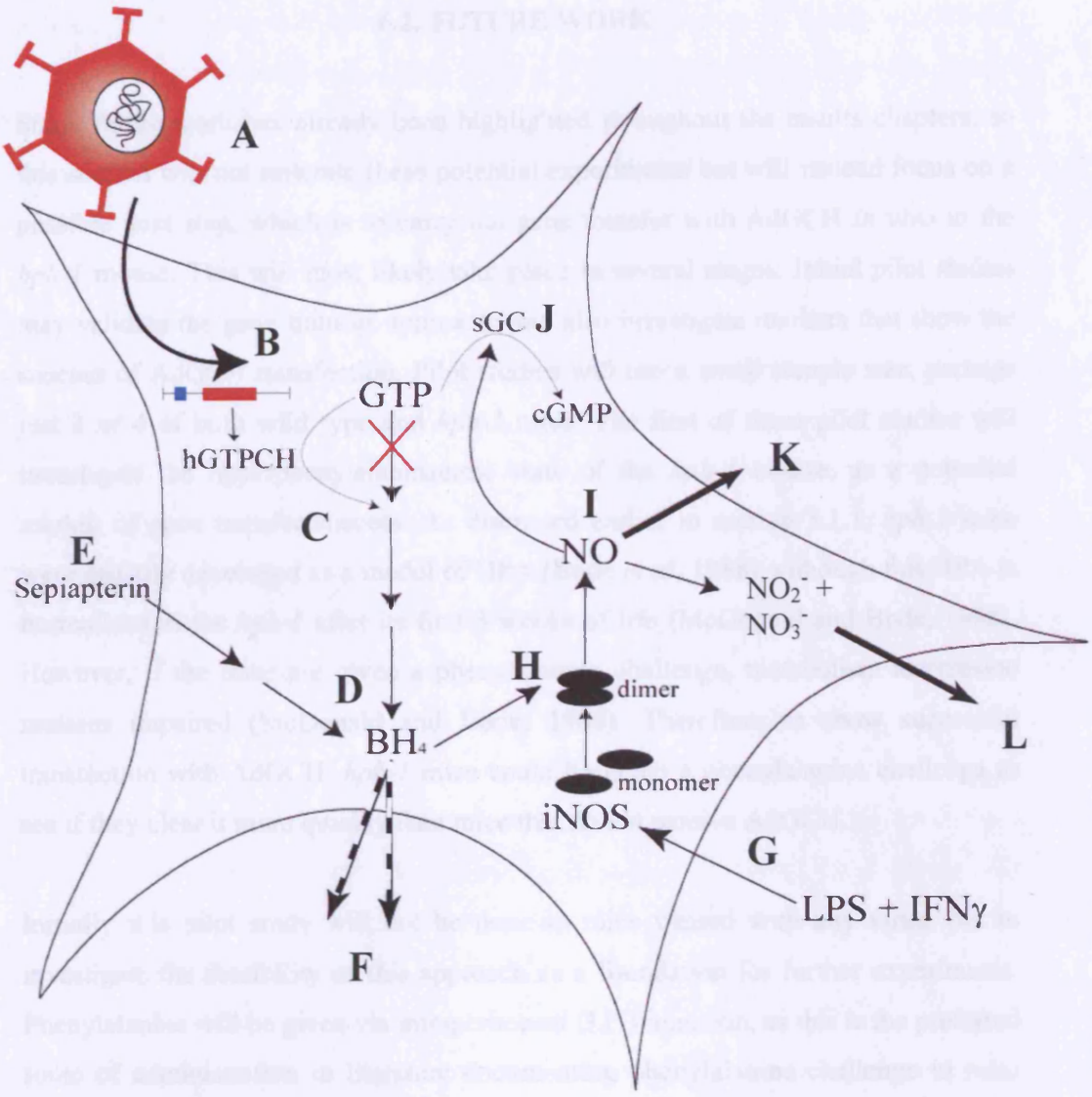


Figure 6.1. Summary diagram of the key findings in *hph-1* astrocytes.

A) AdGCH transfects *hph-1* astrocytes, resulting in B) expression of recombinant human GTP-CH, which C) allows more efficient conversion of GTP to D) BH_4. E) Sepiapterin may also be converted to BH_4 to increase cofactor in *hph-1* cells. Only 1 virus particle per 10 astrocytes is needed to augment BH_4 50-fold, which F) can be taken up by neighbouring cells. BH_4 produced via AdGCH can support LPS + IFN-γ stimulated iNOS expression G). BH_4 assists iNOS dimerisation H), thus increasing NO generation I). NO produced in these cells can then activate sGC to produce cGMP J), be released to other cells, e.g. astrocytes and neurones K), or L) be metabolized to NO_2^- and NO_3^-, and measured in cell culture media.

6.2. FUTURE WORK

Some future work has already been highlighted throughout the results chapters, so this section will not reiterate these potential experiments but will instead focus on a possible next step, which is to carry out gene transfer with AdGCH *in vivo* in the *hph-1* mouse. This will most likely take place in several stages. Initial pilot studies may validate the gene transfer approach, and also investigate markers that show the success of AdGCH transfection. Pilot studies will use a small sample size, perhaps just 3 or 4 of both wild type and *hph-1* mice. The first of these pilot studies will investigate the hyperphenylalaninaemic state of the *hph-1* mouse, as a potential marker of gene transfer success. As discussed earlier in section 3.1.1, *hph-1* mice were initially developed as a model of HPA (Bode *et al*, 1988), although this HPA is normalised in the *hph-1* after its first 3 weeks of life (McDonald and Bode, 1988). However, if the mice are given a phenylalanine challenge, metabolism to tyrosine remains impaired (McDonald and Bode, 1988). Therefore, to show successful transfection with AdGCH, *hph-1* mice could be given a phenylalanine challenge to see if they clear it more quickly than mice that do not receive AdGCH.

Initially this pilot study will not be done in mice treated with any virus, but to investigate the feasibility of this approach as a foundation for further experiments. Phenylalanine will be given via intraperitoneal (I.P.) injection, as this is the preferred route of administration in literature documenting phenylalanine challenge in mice (McDonald and Bode, 1988; Hyland *et al*, 2004). Mice aged 30 days will be used for these experiments, and will be a mixture of male and female, as hyperphenyalaninemia is not documented to be sex related (McDonald and Bode, 1988). Phenylalanine and tyrosine levels can then be measured over a time course of 0-360 minutes post-injection, every 30 minutes in blood from mouse tail snips (McDonald and Bode, 1988). Blood from the tail will be spotted onto filter paper known as a Guthrie card and allowed to dry (Guthrie and Susi, 1963). Then automated electrospray tandem mass spectrometry will be used to measure the amounts of phenylalanine and tyrosine contained in the whole blood (Rashed *et al*, 1997).

A small study has already been carried out on Guthrie blood spots (Guthrie and Susi, 1963), measuring phenylalanine and tyrosine levels in blood spots from decapitated 1-2 day old wild type and *hph-1* mice (table 6.1). Phenylalanine and tyrosine from blood spots were measured by Mr. Geoff Lynes in the Neurometabolic Unit, at the National Hospital for Neurology and Neurosurgery, Queen Square, London, UK using automated electrospray tandem mass spectrometry (Rashed *et al*, 1997). They were quantified using a constant neutral loss scan with internal standards of ring-labelled phenylalanine, and tyrosine-d_4. Phenylalanine concentrations were shown to be significantly elevated in *hph-1* neonates compared to wild type (585.46 ± 58.19 vs. 84.59 ± 7.24nmol/ml; $p < 0.001$, $n = 7\text{-}8$). However, tyrosine concentrations were not different between *hph-1* and wild type (123.83 ± 11.42 vs. 138.23 ± 11.64nmol/ml; $p = 0.50$, $n = 7\text{-}8$). When the ratios of phenylalanine:tyrosine were analysed, the proportion of phenylalanine was significantly higher in *hph-1* mice (4.87 ± 0.48 vs. 0.62 ± 0.04nmol/ml; $p < 0.001$, $n = 7\text{-}8$)(table 6.1). These experiments demonstrate further an impaired ability to metabolise phenylalanine, resulting from a deficiency of BH_4 in the neonatal *hph-1* mouse. This small study also illustrates the feasibility of using small blood volumes on Guthrie cards from *hph-1* mice, to measure phenylalanine and tyrosine levels in blood. Challenging *hph-1* mice with a dose of phenylalanine may then be a valid approach as a marker of the success of AdGCH gene transfer.

Once these experiments looking at the HPA status of the *hph-1* mouse have been conducted, it will be possible to begin treating some of the mice with AdGCH. The most likely route of administration of AdGCH will be via the tail vein, as the liver can take up adenovirus administered by this route, and then potentially act as a source of transgene product for the rest of the organism (Chu, 2004). Of note, BH_4 injected into the periphery (100mg/kg s.c.) can elevate brain levels of BH_4 in *hph-1* mice (Canevari *et al*, 1999). Another option would be to introduce AdGCH into the brain via intracerebroventricular (ICV) injection. This would increase the chances of correcting the brain monoamine, NO and BH_4 deficiencies in the *hph-1* mouse, because the virus could enter the brain, which it would be unlikely to do if injected peripherally. However, ICV injections are both expensive and technically demanding, as they require a cannula to be surgically implanted into the brain of the

	wild type	*hph-1*
Phenylalanine (nmol/ml)	84.59 ± 7.24	585.46 ± 58.19*
Tyrosine (nmol/ml)	138.23 ± 11.64	123.83 ± 11.42
Phenylalanine:Tyrosine Ratio	0.62 ± 0.04	4.87 ± 0.48$

Table 6.1. Comparison of the levels of phenylalanine, tyrosine and the phenylalanine to tyrosine ratio in wild type versus *hph-1* whole blood. Whole blood was collected onto a Guthrie card, as described by the method of Guthrie and Susi, 1963. Phenylalanine and tyrosine were measured by Mr. Geoff Lynes in the Neurometabolic Unit, at the National Hospital for Neurology and Neurosurgery, Queen Square, London, using automated electrospray tandem mass spectrometry. Data is expressed as mean ± SEM (n = 7-8). * = $p < 0.001$ vs. wild-type, as judged using Student's t-test. $ = $p < 0.001$ vs. wild-type, as determined first by transformation of ratios for statistical analysis (section 2.10.), followed by one way ANOVA followed by least significant difference test.

animal (Gupta and Bhargava, 1965); a method that is made more challenging in a small rodent such as a mouse.

Before larger scale experiments can begin, another set of pilot studies will need to examine which dose of virus is acceptable for treatment. A therapeutic window must be found where functional effects are observed, but toxicity is minimal. One of the drawbacks of using adenoviruses as vectors for gene transfer is the severe innate immune response they can trigger. This can limit their survival in host cells and their use in gene therapy (Byrnes *et al*, 1995; van Ginkel *et al*, 1997). With these toxicity issues in mind, Dr. Shijie Cai has been developing an adeno-associated virus, which will contain human GTP-CH like AdGCH. This may reduce toxicity and be better tolerated by the animals. However, at present this remains in development.

Once a safe and efficacious dose of virus is found, it is possible to begin experiments to validate the success of the gene transfer. Parameters that would need to be measured include BH_4 levels in brain, liver and also plasma; NO_2^- and NO_3^- in brain and plasma, as well as cGMP and monoamine neurotransmitters in the brain. Expression of recombinant GTP-CH in liver and brain would be analysed using Western blotting for the anti-HA epitope, and visualization of AdeGFP in liver slices would provide more evidence of gene transfer.

An alternative approach to correcting the GTP-CH deficiency in *hph-1* mice, is to administer AdGCH to neonatal pups, aged between 1-5 days. The advantage of this approach is that the HPA *hph-1* mice display before 3 weeks old (McDonald and Bode, 1988) can be used as a marker of gene transfer success, rather than dosing animals with phenylalanine. Furthermore, gene transfer in neonatal mice may be more successful than in adult mice, as the neonatal immune system and blood-brain barrier is less developed, and adenoviral gene transfer has been shown to be more effective in neonates (Gilchrist *et al*, 2002; Saunders *et al*, 1999). One of the major drawbacks of using adenoviral vectors is the immune response they can trigger (Kafri *et al*, 1998), so dosing neonatal mice may lead to fewer immunological consequences.

However, using mice so young can also have drawbacks. To begin with, measuring phenylalanine requires obtaining blood from the mice, which may be problematic in such young animals, as the volume of blood that can be taken is very low, unless blood is obtained from decapitation as described above and shown in table 6.1.

However, this requires killing the animal, so monitoring the longer-term effects of gene transfer is not then possible. An alternative method of taking blood from neonates is from tail snips, but this limits the amount volume of blood that can be removed, which may not be sufficient for accurate determination of phenylalanine and tyrosine concentrations. However, pilot studies would reveal whether this technical difficulty might be an obstruction to conducting full gene transfer studies in neonatal *hph-1* mice.

The route of administration of AdGCH must also be taken into account in neonatal mice. Injecting virus via the tail vein may prove to be technically difficult, due to the size of the blood vessel, so an alternative may be intramuscular (i.m.) injection or s.c. injection (Gilchrist *et al*, 2002). If this is successful then the mice would be left to develop, and markers of gene transfer success including BH_4, NO and monoamine metabolism, and recombinant GTP-CH expression, could be measured over various time-points to monitor correction of the defect.

Taken together, these two different approaches to *in vivo* gene transfer of AdGCH in neonatal and adult mice, are potential methods to correct the impaired BH_4 metabolism in the *hph-1* mouse. If either or both of these approaches are successful then perhaps ultimately a viral vector containing recombinant human GTP-CH could be used for gene therapy in humans suffering from impaired BH_4 metabolism.

Chapter 7

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7.0. REFERENCES

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