The neurochemical consequences of aromatic L-amino acid decarboxylase deficiency

Submitted By:

George Francis Gray Allen

Department of Molecular Neuroscience

UCL Institute of Neurology

Queen Square, London

Submitted November 2010

Funded by the AADC Research Trust, UK

Thesis submitted for the degree of Doctor of Philosophy, University College London (UCL)

I, George Allen confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed…….Date……

Abstract

Aromatic L-amino acid decarboxylase (AADC) catalyses the conversion of 5-hydroxytryptophan (5-HTP) and L-3,4-dihydroxyphenylalanine (L-dopa) to the neurotransmitters serotonin and dopamine respectively. The inherited disorder AADC deficiency leads to a severe deficit of serotonin and dopamine as well as an accumulation of 5-HTP and L-dopa. This thesis investigated the potential role of 5-HTP/L-dopa accumulation in the pathogenesis of AADC deficiency. Treatment of human neuroblastoma cells with L-dopa or dopamine was found to increase intracellular levels of the antioxidant reduced glutathione (GSH). However inhibiting AADC prevented the GSH increase induced by L-dopa. Furthermore dopamine but not L-dopa, increased GSH release from human astrocytoma cells, which do not express AADC activity. GSH release is the first stage of GSH trafficking from astrocytes to neurons. This data indicates dopamine may play a role in controlling brain GSH levels and consequently antioxidant status. The inability of L-dopa to influence GSH concentrations in the absence of AADC or with AADC inhibited indicates GSH trafficking/metabolism may be compromised in AADC deficiency. 5-HTP was demonstrated to potentially be mildly toxic to human neuroblastoma cells but not astrocytoma cells; however the concentrations required for this response are likely to be higher than pathophysiological levels in AADC deficiency. These results indicate the need for investigations addressing the effects of chronic 5-HTP exposure as only acute effects were investigated in the current study. This thesis also investigated the effect of altered availability of the AADC coenzyme pyridoxal 5'-phosphate (PLP) on AADC activity, protein and expression. In two patients with inherited disorders of PLP metabolism reductions in plasma AADC activity were observed. Furthermore PLP-deficient human neuroblastoma cells were found to exhibit reduced levels of AADC activity and protein but not altered expression. These findings suggest maintaining adequate PLP availability may be important for optimal treatment of AADC deficiency.

Table of Contents

Table of Figures

List of Tables

List of Abbreviations

3-OMD	3-O-methyldopa
4-DP	4-deoxypyridoxine
5-HIAA	5-hydroxyindoleacetic acid
5-HTP	L-5-hydroxytryptophan
5-MTHF	5-methyltetrahydrofolate
AADC	aromatic L-amino acid decarboxylase
AC	adenylyl cyclase
ADP	adenosine diphosphate
ALDH	aldehyde dehydrogenase
alt-DDC	alt-dopa decarboxylase
ApN	aminopeptidase N
AREs	antioxidant response elements
ATP	adenosine triphosphate
$-B_6$	pyridoxine deficient medium
BH_4	tetrahydrobiopterin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNS	central nevous system
COMT	catechol O-methyltransferase
COMTI	catechol-O-methyltransferase inhibitor
CSF	cerebrospinal fluid
CuZnSOD	copper and zinc containing superoxide dismutase
CysGly	cysteinylglycine
DAG	diacylglycerol
DAT	dopamine transporter
DHPR	dihydropteridine reductase
DMEM	Dulbecco's modified Eagle's medium
DMEM/F-12	Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture
DNTB	5',5'-dithio-bis(2-nitrobenzoic acid)
DOPAC	3,4-dihydroxyphenylacetic acid
DβH	dopamine β-hydroxylase
ECD	electrochemical detection
EDTA	ethylenediaminetetrahydroacetic acid
FAD	flavine adenine dinucleotide
GABA	γ-amino butyric acid
GAPDH	glyceraldehydes 3'-phosphate dehydrogenase
GPCR	g-protein coupled receptor
GPx	glutathione peroxidase
GS	glutathione synthase
GSH	reduced glutathione
GSSG	glutathione disulphide
GTP	guanosine triphosphate
GTPCH	guanosine triphosphate cyclohydrolase
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography

HRP	horseradish peroxidase
HVA	homovanillic acid
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-γ
iNOS	inducibe nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate
KCN	potassium cyanide
Keap1	kelch-like ECH associating protein 1
LDH	lactate dehydrogenase
L-dopa	L-3,4-dihydroxyphenylalanine
LGIC	ligand-gated ion channel
LSD	least significant difference
MA	monoamine
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MnSOD	manganese containing superoxide dismutase
MRP1	multidrug resistance protein 1
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl D-aspartate
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
NQO1	NAD(P)H quinine oxidaoreductase
Nrf2	nuclear factor erythroid 2-related factor
NSD-1015	3-hydroxybenzylhydrazine dihydrochloride
PBST	phosphate buffered saline 0.5mL/L Tween 20
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKE	pig kidney epithelial cells
PLC-β	phospholipase C-β
PLP	pyridoxal 5'-phosphate
PNMT	phenylethanolamine N-methyltransferase
PNPO	pyridox(am)ine 5'-phosphate oxidase
PTPS	6-pyruvoyltetrahydropterin synthase
PVDF	polyvinylidene fluoride
RIP140	receptor interacting protein 140
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide electrophoresis
SEM	standard error of the mean
SERT	serotonin transporter
SNc	substantia nigra pars compacta
SOD	superoxide dismutase
SRB	sulforhodamine B
TAT	tyrosine aminotransferase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
VLA	vanillactic acid

VMAT1	vesicular monoamine transporter 1
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area
α-KAR	α-ketoacid reductase
γ-GCL	γ-glutamylcysteine ligase
γ-GT	γ-glutamyl transpeptidase

Acknowledgements

I would like to thank my principle supervisor Prof. Simon Heales, his knowledge and guidance has been invaluable, his passion and commitment to science has been infectious and his balance between work and play has made my entire PhD experience enjoyable.

I am grateful also to my subsidiary supervisor Dr. John Land for his supervision, advice and particularly for his exceptional eye for detail and encyclopaedic knowledge of biochemistry and medicine.

This thesis would not have been possible without the financial support of the AADC Research Trust, its board and to all those who have fundraised for them, I am indebted to you all. In particular the enthuisum and commitment of the managing director Lisa Flint to improving the understanding and treatment of AADC deficiency has provided this work with both a driving force and an inspiration.

My thanks go to Dr. Keith Hyland for determining the AADC kinetics of a patient with pyridox(am)ine 5'-phosphate oxidase deficiency. I would also like to thank Dr. Iain Hargreaves for his help with mitochondrial respiratory chain activity assays as well as for all his advice, help and suggestions throughout this study. Thanks also go to Viruna Neergheen and Marcus Oppenheim (Neurometabolic Unit, National Hospital) for measuring pyridoxal 5'-phosphate levels.

I would like to thank every member of the Neurometabolic Unit for all their help throughout my PhD, particularly when I have come scrounging for HPLC spares.

I am grateful to all those at the Institute of Child Health who have been involved with this project especially Prof. Peter Clayton, Dr. Emma Footitt and Dr. Philippa Mills.

My thanks also go to each member of the Department of Molecular Neuroscience and especially to Dr. Julia Fitzgerald, Laura Dunn, Dr. Lee Stanyer, Abi Li and Kate Duberley.

My parents Cliff and Sue Allen deserve a special mention, I am especially grateful for your belief and support; it has provided the foundation that has allowed me to undertake this work.

For their support and encouragement I would also like to thank my sister Rachel Hogarth and her husband Ed Hogarth, My grandparents Peter and Dorie Allen, and Peter and Marie Gray, and all of my family.

Thanks also go to Jerome Cheung, Daniel Valko, Phil Hodgekin, Alex Cookson, Naomi Cochrane, Chris Miller, Jude O'Connor, Reena Hill and Phil Adams.

Finally to Rachel Winzer, thank you for your help, for your patience and for your encouragement. I could not have done this without you.

Chapter 1

Introduction

1.1 Monoamine Neurotransmitters

Serotonin and dopamine are aromatic amines derived from the decarboxylation of the amino acids L-5-hydroxytryptophan (5-HTP) and L-3,4-dihydroxyphenylalanine (L-dopa) respectively, by the enzyme aromatic L-amino acid decarboxylase (AADC; EC No. 4.1.1.28). These two amines act as extracellular molecular signals within the nervous system and in peripheral tissues. Both serotonin and dopamine have been demonstrated to participate in a range of physiological and behavioural processes. Serotonin was first identified and purified as a substance in platelets and serum with constrictor activity of smooth muscle, including blood vessels and duodenum (Zucker *et al*, 1944; Rapport *et al*, 1948). Subsequently serotonin was found to be present in rodent and dog brain and was consequently suggested to act as a neurotransmitter (Twarog *et al*, 1953; Amin *et al*, 1954). Dopamine was first isolated from sheep heart and adrenal medulla, but was considered to act primarily as the immediate precursor of noradrenaline (Goodall, 1951). However it was subsequently demonstrated that administered dopamine could reduce arterial blood pressure in guinea pig (Hornykiewicz, 1958). Furthermore enhanced concentrations of dopamine, but not noradrenaline, in the brain were found to reverse the tranquilizing effect of reserpine in rodents (Carlsson *et al*, 1957; Carlsson *et al*, 1958; Everett and Weigand, 1962). This latter evidence indicated that dopamine was also a neurotransmitter and that it was involved in motor behaviour (reviewed by Hornykiewicz, 1966).

1.1.1 Serotonin

Serotonin has been demonstrated to play a complex modulatory role in a wide range of behavioural functions (reviewed by Jacobs and Fornal, 1997a; Lucki, 1998). Central serotonin is considered to be important for the induction and maintenance of sleep such that pharmacological depletion of serotonin has been shown to disturb sleep or reduce some stages of sleep (Koella *et al*, 1968; Wyatt *et al*, 1969; Sheard, 1969). Eating behaviour is also thought to be modulated by serotonin (reviewed by Simansky, 1996). Serotonin reuptake inhibitors and non-selective serotonin agonists have been found to reduce food intake and induce satiety while serotonin depletion can produce hyperphagia and weight gain (Breisch *et al*, 1976; Saller and Stricker, 1976; Blundell and Latham, 1980; Simansky and Vaidya, 1990). This effect may in part relate to serotonergic modulation of neuroendocrine functions such as release of melanocortins (Heisler *et al*, 2002; Heisler *et al*, 2006). Moreover serotonin is known to play a

complex role in the control of mood and emotion, for example serotonin depletion enhances cognitive and behavioural responses to punishment or fearful stimuli whilst also reducing the ability to modify behaviour to avoid punishment (Cools *et al*, 2005; van der Veen *et al*, 2007; Cools *et al*, 2008; Crockett *et al*, 2009). Further roles of central serotonin include modulation of body temperature, movement and memory (Yamada *et al*, 1988; Fornal *et al*, 1996; Jacobs and Fornal, 1997b, Stewart *et al*, 1997; Riedel *et al*, 1999; Molodtsova, 2008).

1.1.2 Dopamine

Dopaminergic pathways in the brain are considered to be involved in a range of behavioural and cognitive functions. The neuronal dopamine pathway originating in the substantia nigra pars compacta and terminating in the corpus striatum is known to play an essential role in the basal-ganglia motor circuits that are involved in the planning and execution of voluntary movement (reviewed by Mink and Thach, 1993). This is perhaps best demonstrated by the primary pathology of Parkinson's disease which involves the degeneration of this dopaminergic pathway leading to the characteristic clinical Parkinsonian features of hypokinesia, rigidity and tremor (Hassler, 1938; Hornykiewicz, 1963; Dahlstrom and Fuxe, 1964; Fuxe, 1965). Dopamine is also thought to be involved in learning and working memory (reviewed by Robbins and Everitt, 2002). Dopamine antagonists have been found to impair spatial working memory and planning whilst dopamine agonists can improve some aspects of working memory (Luciana *et al*, 1992; Luciana *et al*, 1997; Mehta *et al*, 1999). In addition dopamine is considered to modulate reward and motivational behaviour, which can be disrupted with dopamine antagonists (Wise *et al*, 1978; Beninger and Hahn, 1983; Beninger *et al*, 1987). Central dopamine may also be involved in the modulation of the sleep-wake cycle (Monti, 1968; Kafi and Gaillard, 1976) and hypothalamic dopamine is known to be involved in the inhibition of prolactin and melanocyte stimulating hormone secretion (Caron *et al*, 1978; Munemura *et al*, 1980).

1.2 Monoamine Metabolism

1.2.1 Indoleamine Metabolism

Both serotonin and dopamine are formed from the essential aromatic amino acids L-tryptophan and L-phenylalanine respectively. Serotonin is produced from L-tryptophan by two sequential steps (see figure 1.1). L-tryptophan is hydroxylated to 5-HTP by

tryptophan hydroxylase (EC No. 1.14.16.4) requiring tetrahydrobiopterin (BH_4) and O_2 (Grahame-Smith, 1964; Green and Sawyer, 1966; Friedman *et al*, 1972). The hydroxylation of L-tryptophan is considered to be rate limiting for serotonin synthesis, although the availability of L-tryptophan can also influence synthesis rate (Neckers *et al*, 1977). Subsequently 5-HTP is decarboxylated to serotonin by AADC which requires pyridoxal 5'-phosphate (PLP) as a co-enzyme (Lovenberg *et al*, 1962). In melatonin producing cells such as in the pineal gland serotonin undergoes acetylation to N-acetylserotonin by arylamine N-acetyltransferase (EC No. 2.3.1.5) using acetyl coenzyme A as a cofactor and subsequently methylated by acetylserotonin-*O*-methyltransferase (EC No. 2.1.1.4) to melatonin with S-adenosylmethionine (SAM) as a methyl donor (Weissbach *et al*, 1960; Axelrod and Weissbach, 1960).

1.2.2 Catecholamine Metabolism

L-phenylalanine is hydroxylated at the 4-position of the phenyl group to produce L-tyrosine by the enzyme phenylalanine hydroxylase (EC No. 1.14.16.1) that requires BH_4 and O_2 (Udenfriend and Cooper, 1952; Mitoma, 1956, Kaufman, 1959). The subsequent hydroxylation of L-tyrosine at position-3 of the phenyl group to give L-dopa is catalysed by tyrosine hydroxylase (EC No. 1.14.16.2). This is the rate limiting step for dopamine synthesis and also requires BH_4 and O_2 (see figure 1.2; Hess *et al*, 1961; Nagatsu *et al*, 1964). L-dopa is then decarboxylated by AADC to dopamine, again requiring PLP (Holtz *et al*, 1938; Holtz *et al*, 1939). In noradrenergic cells dopamine is further hydroxylated to noradrenaline by dopamine β-hydroxylase (EC No. 1.14.17.1) in a reaction requiring ascorbate and O_2 (Levin *et al*, 1960), and in adrenergic cells noradrenaline is methylated by phenylethanolamine N-methyltransferase (EC No. 2.1.1.28) to adrenaline using S-adenosylmethionine as a methyl donor (Axelrod, 1962).

1.3 Monoamine Neurotransmission

1.3.1 Vesicular Uptake

Following production within neurons or neuroendocrine cells the monoamine neurotransmitters are packaged into small synaptic vesicles or dense core secretory vesicles. Uptake of monoamines into vesicles is catalysed by vesicular monoamine transporters of which there are two forms termed vesicular monoamine transporter 1 (VMAT1) and vesicular monoamine transporter 2 (VMAT2; see figure 1.3; Liu *et al*, 1992; Erickson *et al*, 1992). VMAT2 is expressed in both central and peripheral

neurons while VMAT1 is expressed mainly in the adrenal medulla and endocrine cells of the gastrointestinal tract (Peter *et al*, 1995). It has recently been demonstrated that VMAT2 forms a complex with AADC and TH in rat striatal neurons suggesting that there may be integration of dopamine metabolism and vesicular storage (Cartier *et al*, 2010). The K_m apparent for the vesicular uptake of dopamine and serotonin respectively were measured as 21.0μM and 0.6μM (Gershten *et al*, 1983; Ruth *et al*, 1986). These K_m values are at least 10-fold lower than those for AADC (see section 1.6.3) indicating that, following production, vesicular uptake of monoamines has the potential to be highly efficient. The uptake of monoamines into vesicles is driven by a proton gradient whereby one molecule of monoamine in cationic form is exchanged for $2H^+$ (Knoth *et al*, 1980; Daniels and Reinhard, 1988; Darchen *et al*, 1988). The proton gradient is established by the vacuolar-type H^+ ATPase proton pump (Xie and Stone, 1986; Moriyama and Nelson, 1987; Cidon and Sihra, 1989).

1.3.2 Release

Once filled with neurotransmitter, synaptic vesicles translocate to the active zone of the synapse, the presynaptic area directly adjacent to the synaptic cleft. Vesicles make contact with the plasma membrane in a process referred to as docking, and subsequently become primed for neurotransmitter release (reviewed by Sudhof, 2004; Wojcik and Brose, 2007). Priming is thought to involve the assembly of a complex of proteins including the SNARE proteins that collectively form the machinery for membrane fusion (Sollner *et al*, 1993; McMahon *et al*, 1995). Upon membrane depolarisation, due to an action potential, voltage gated Ca^{2+} channels within the active zone open leading to Ca^{2+} influx that triggers vesicle exocytosis. Ca^{2+} evoked exocytosis most likely involves synaptotagmin proteins which are considered to act as Ca^{2+} sensors and that also interact with the membrane fusion protein complex (Geppert *et al*, 1994; Reim *et al*, 2001). Following neurotransmitter release vesicles undergo endocytosis and are recycled either directly or via endosomes (Ceccarelli *et al*, 1973; Heuser and Reese, 1973; Sudhof, 2004).

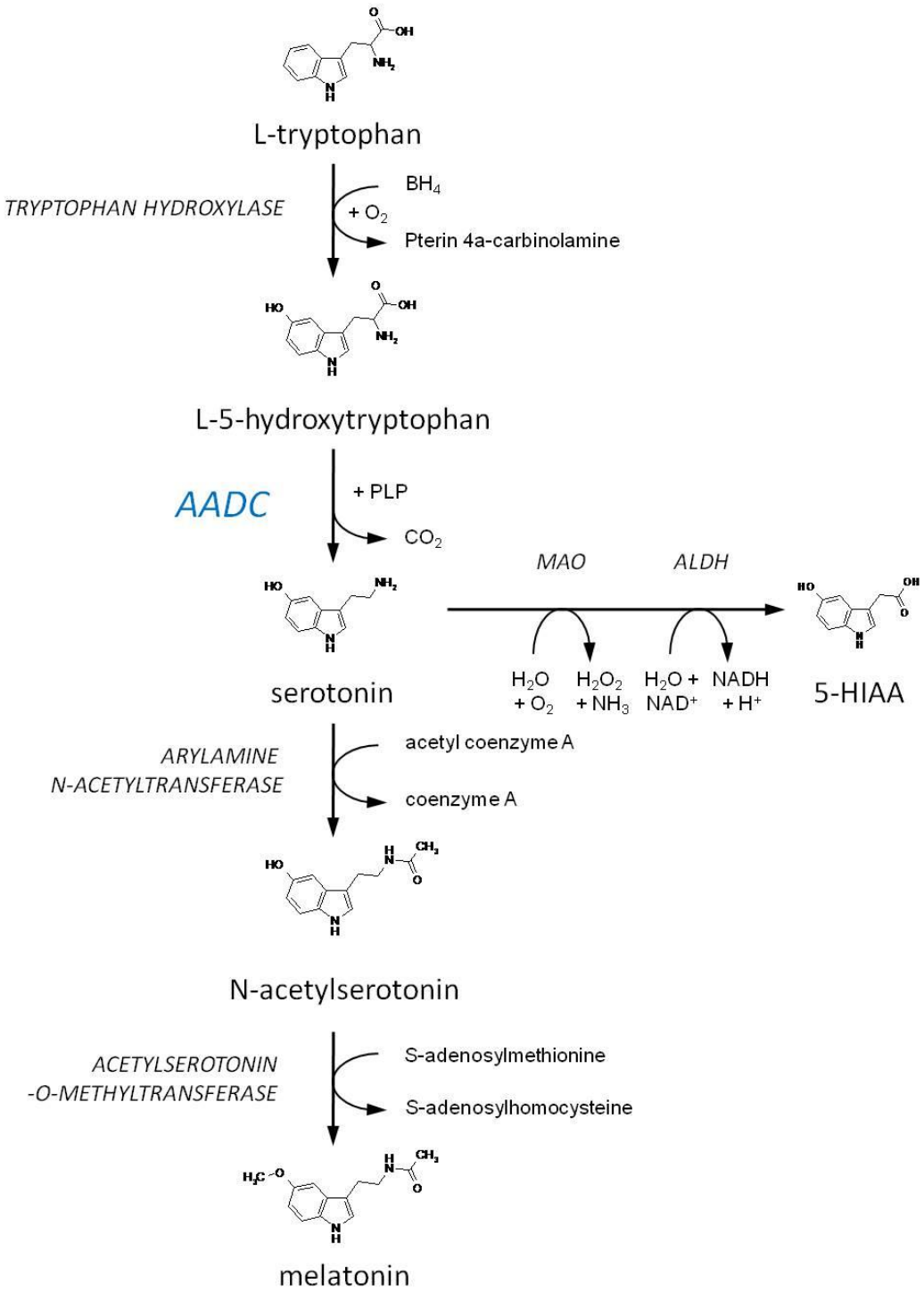


Figure 1.1 Indoleamine metabolic pathway. BH_4: tetrahydrobiotperin, AADC: aromatic L-amino acid decarboxylase, PLP: pyridoxal 5'-phosphate, MAO: monoamine oxidase, ALDH: aldehyde dehydrogenase, 5-HIAA: 5-hydroxyindoleacetic acid.

1.3.3 Receptors

Serotonin or dopamine released into the synaptic cleft bind to specific receptors located at the post-synaptic membrane, but also to receptors at the presynaptic membrane, termed autoreceptors. The serotonin receptors are grouped into seven families termed 5-HT_{1-7} with between one and five receptors in each family (reviewed by Hartig, 1997). All serotonin receptors are G-protein coupled receptors (GPCRs) except for the 5-HT_3 receptors which are ligand-gated ion channels. The 5-HT_3 receptor ion channels are cation selective and when activated lead to membrane depolarisation (Derkach *et al*, 1989). There are two families of dopamine receptor, the D_1-like receptors and the D_2-like receptors, of which D_1 and D_5 belong to the former and D_2, D_3 and D_4 belong to the latter (reviewed by Missale *et al*, 1998). All dopamine receptors are GPCRs. In an unactivated state GPCRs are associated with specific G-proteins. Upon receptor activation the G-protein becomes dissociated from the receptor and can then activate multiple intracellular proteins, termed effectors (reviewed by Gilman, 1987). Each serotonin and dopamine GPCR is linked to a specific range of effector proteins. It is important to note that different serotonin or dopamine receptors may have opposing intracellular actions. For example D_1-like receptors stimulate cyclic adenosine monophosphate (cAMP) production, whilst D_2-like receptors inhibit cAMP production (Kebabian and Calne, 1979; Onali *et al*, 1984; Picetti *et al*, 1997). In addition to the diversity of intracellular signalling, each dopamine and serotonin receptor has a unique central and peripheral distribution (Weiner *et al*, 1991; Tecott *et al*, 1993; Pompeiano *et al*, 1994). This complexity of receptor distribution and intracellular signalling may underlie some of the difficulties in targeting these receptors for disease treatment (see section 1.5.4).

1.3.4 Re-uptake

Neurotransmitter released into the synaptic cleft can be sequestered back into the presynaptic nerve terminal. This process is achieved in the case of serotonin and dopamine by specific membrane transporter proteins, respectively termed the serotonin transporter (SERT) and the dopamine transporter (DAT; Kilty *et al*, 1991; Shimada *et al*, 1991; Blakely *et al*, 1991; Hoffman *et al*, 1991). At the axon terminal electron microscopy indicates that SERT and DAT are located mainly at the plasma membrane but not directly positioned within the synaptic cleft, suggesting that serotonin and dopamine diffuse out of the synaptic cleft prior to re-uptake (Nirenberg *et al*, 1996;

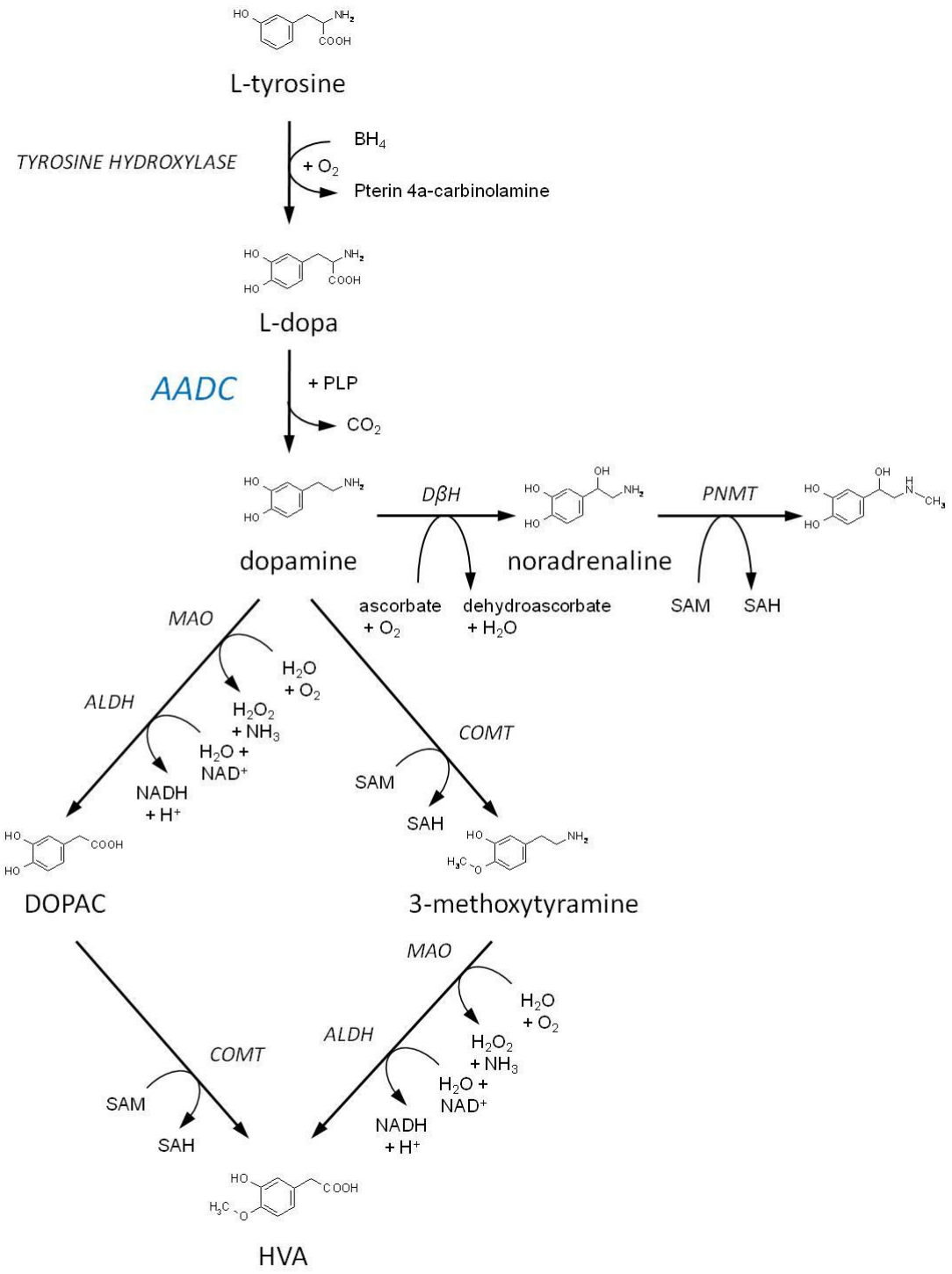


Figure 1.2 catecholamine metabolic pathway. BH_4: tetrahydrobiotperin, AADC: aromatic L-amino acid decarboxylase, L-dopa: L-3,4-dihydroxyphenylalanine, PLP: pyridoxal 5'-phosphate, DβH: dopamine β-hydroxylase, PNMT: phenolethanolamine N-methyltransferase, SAM: S- adenosylmethionine, SAH: S-adenosylhomocysteine, MAO: monoamine oxidase, ALDH: aldehyde dehydrogenase, COMT: Catechol O-methyltranserafe, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: Homovanillic acid.

Zhou *et al*, 1998). Transport across the plasma membrane is driven by the Na^+ and Cl^- concentration gradient. For serotonin one Na^+ ion and one Cl^- ion are co-transported,

whilst dopamine is co-transported with two Na^+ ions and one Cl^- ion (Gu *et al*, 1994). Similar to vesicular uptake the ion concentration gradient is energetically established via an ATPase pump, in this case the plasma membrane Na^+/K^+ ATPase (Dunham and Glynn, 1961; Tissari *et al*, 1969). Once returned to the presynaptic terminal dopamine and serotonin can be taken back up into vesicles. SERT and DAT knock-out mice have reduced tissue concentrations of serotonin or dopamine respectively, but extracellular concentrations are increased (Giros *et al*, 1996; Bengel *et al*, 1998). This suggests that not only is re-uptake the major mechanism for clearance of extracellular monoamine neurotransmitters but also that reuptake makes a substantial contribution to the levels of stored serotonin and dopamine. However tyrosine hydroxylase was down-regulated in the DAT knock-out mouse which may suggest that the decrease in stored dopamine may have been related to decreased synthesis as well as to reduced recycling.

1.3.5 Degradation

Serotonin or dopamine that is not recycled back into vesicles is degraded either within neurons or glial cells (Levitt *et al*, 1982). The initial step in serotonin breakdown is catalysed by monoamine oxidase (MAO; EC No. 1.4.3.4; see figure 1.1) that exists in two isoforms termed MAO-A and MAO-B derived from different genes (Bach et al, 1988). MAO oxidises serotonin to 5-hydroxyindoleacetaldehyde, requiring H_2O and O_2 (Sjoerdsma *et al*, 1955). This product is then dehydrogenated to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH; EC No. 1.2.1.3) with oxidised nicotinamide adenine dinucleotide (NAD^+; Maring *et al*, 1985). Dopamine can be broken down by two alternative metabolic routes both producing homovanillic acid (HVA; see figure 1.2). Firstly dopamine can be converted to dihydroxyphenylacetaldehyde by MAO, which is rapidly converted to dihydroxyphenylacetic acid (DOPAC) by ALDH with NAD^+, and is then converted to HVA by catechol-*O*-methyltransferase (COMT; EC No. 2.1.1.6) using SAM as a methyl donor (Rosengren, 1960; Maring *et al*, 1985). Alternatively COMT can metabolise dopamine to 3-methoxytyramine and subsequently MAO and ALDH convert 3-methoxytyramine to HVA (Axelrod and Tomchick, 1958).

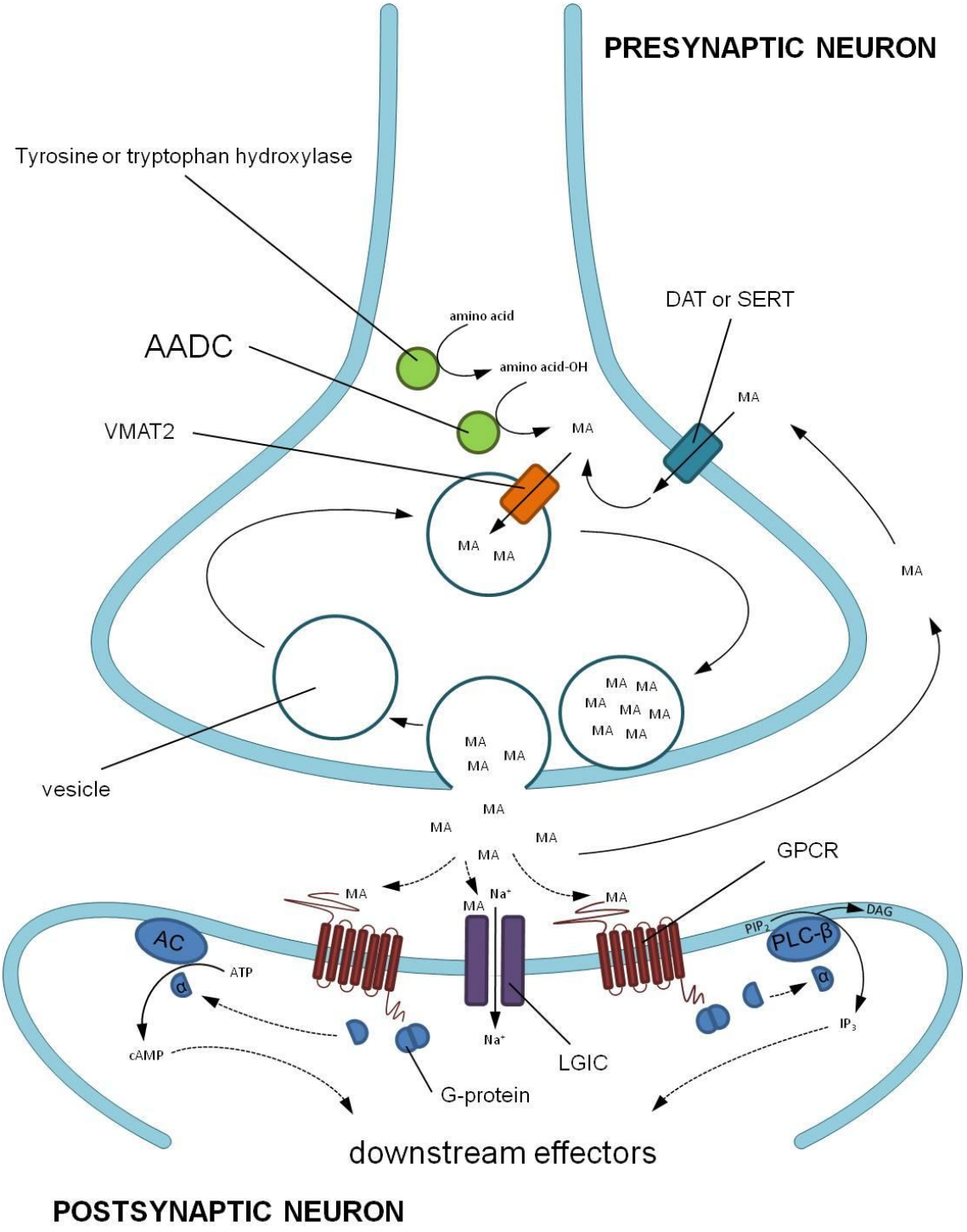


Figure 1.3 Monoamine neurotransmission. MA: monoamine, AADC: aromatic L-amino acid decarboxylase, VMAT2 vesicular monoamine transporter 2, DAT: dopamine transporter, SERT: serotonin transporter, GPCR: g-protein coupled receptor, LGIC: ligand-gated ion channel, AC: adenylyl cyclase, PLC-β: phospholipase C-β, PIP_2: phosphatidylinositol 4,5-bisphosphate, DAG: diacylglycerol, IP_3: inositol 1,4,5-triphosphate.

1.4 Disorders of Aromatic Amino Acid Metabolism

1.4.1 Hyperphenyalaninemia

The first disorder affecting the metabolism of an aromatic amino acid to be described was phenylalanine hydroxylase deficiency (Folling, 1934; Penrose, 1935). This condition was originally recognised by the high urinary excretion levels of phenylpyruvic acid, phenylketonuria, found in some patients with severe mental retardation. Controlling the hyperphenylalaninemia in these patients, by restricting phenylalanine intake, was found to be an effective treatment, leading to some reversal of cognitive deficits, hence in this disorder the accumulation of precursor appears to be the primary pathogenic mechanism (see section 1.7.1; Bickel *et al*, 1953; Woolf *et al*, 1955; Blainey and Gulliford, 1956). The subsequent realisation that hyperphenylalaninemia was not exclusive to phenylalanine hydroxylase deficiency led to the discovery of the deficiencies of BH_4 metabolism, the cofactor for phenylalanine hydroxylase (Kaufman *et al*, 1975; see figure 1.4). Disorders of BH_4 metabolism have been described for both the enzymes involved in BH_4 recycling, 4a-carbinolamine dehydratase and dihydropteridine reductase (DHPR), as well as those involved in BH_4 synthesis, guanosine triphosphate cyclohydrolase (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (Kaufman *et al*, 1975; Neiderwieser *et al*, 1984; Niederwieser *et al*, 1985; Citron *et al*, 1993; Bonafe *et al*, 2001a). With the exception of sepiapterin reductase deficiency and the dominant form of GTPCH deficiency all of these conditions present with hyperphenylalaninemia. For those BH_4 conditions with hyperphenylalaninemia correcting phenylalanine levels forms an essential part of treatment, however as BH_4 also acts as a cofactor for tyrosine hydroxylase and tryptophan hydroxylase most forms of BH_4 deficiency also present with a severe deficit of serotonin and dopamine (Hyland, 1999; Blau, 2004; Longo, 2009).

1.4.2 Monoamine Neurotransmitter Deficiency

A near absence of serotonin and dopamine can be caused by a deficiency of AADC, which converts 5-HTP to serotonin and L-dopa to dopamine (Hyland and Clayton, 1990; Hyland *et al*, 1992; see section 1.5). An inherited deficiency of tyrosine hydroxylase, which converts L-tyrosine to L-dopa, has also been described, which leads to dopamine deficiency with serotonin metabolism unaffected (Ludecke *et al*, 1996). In severe forms of autosomal recessive GTPCH, PTPS and DHPR deficiency the common

clinical symptoms include mental retardation, convulsions, hypokinesia, oculogyric crises, truncal hypotonia, ptosis, irritability, hypersalivation, temperature instability and excessive sweating (Hyland, 1999; Blau, 2004). The majority of these symptoms are also shared with AADC deficiency except convulsions which are generally considered uncommon in AADC deficiency (Swoboda *et al*, 1999; Swoboda *et al*, 2003; Pons *et al*, 2004; Brun *et al*, 2010). Clinical features of tyrosine hydroxylase deficiency include psychomotor delay, truncal hypotonia, hypokinesia, dystonia, rigidity, tremor, oculogyric crises, ptosis and hypersalivation (Hoffmann *et al*, 2003). Ptosis, hypersalivation and excessive sweating are commonly attributed to the consequential autonomic dysfunction of noradrenaline and adrenaline deficiency (Swoboda *et al*, 2003; Brun *et al*, 2010). Movement related deficits are considered to be largely due to dopamine deficiency, however in AADC deficiency and BH_4 deficiencies there may also be a serotonin component, as serotonergic neurons are known to modulate some motor functions (Fornal *et al*, 1996; Jacobs and Fornal, 1997b; Swoboda *et al*, 2003; Brun *et al*, 2010). The aetiology of oculogyric crises is not well understood, however as they occur in AADC deficiency, BH_4 deficiencies and also tyrosine hydroxylase deficiency it is likely to involve catecholamine deficiency, rather than indoleamine deficiency. Furthermore as oculogyric crises have not been reported in dopamine β-hydroxylase deficiency (Robertson *et al*, 1991) this could suggest that they are specifically related to dopamine deficiency. Serotonin deficiency may manifest as relatively subtle but wide ranging functional alterations due to its complex but largely modulatory role in many functions. Examples of symptoms likely to involve serotonin include sleeping difficulties, temperature instability and problems with mood (Swoboda *et al*, 2003; Brun *et al*, 2010).

1.4.3 Treatment

GTPCH and PTPS deficiency are treated with BH_4. BH_4 does not easily cross the blood brain barrier and consequently its administration is aimed largely at reducing blood phenylalanine levels rather than correcting monoamine neurotransmitter defects (Hyland, 2007; Longo, 2009). Serotonin and dopamine also do not cross the blood brain barrier. Instead, in an attempt to correct the monoamine neurotransmitter deficiency 5-HTP and L-dopa are administered (Shintaku, 2002; Blau, 2004). This treatment bypasses the metabolic block at tyrosine and tryptophan hydroxylases and administered 5-HTP and L-dopa are converted by AADC in the brain to serotonin and

dopamine. For tyrosine hydroxylase deficiency the main treatment is L-dopa (Hoffmann *et al*, 2003). Treatment of these conditions with 5-HTP and L-dopa is dependent on functioning central AADC, accordingly most cases of AADC deficiency cannot be treated in this manner (see section 1.5.4). Treatment with 5-HTP and/or L-dopa is commonly accompanied by administration of a peripheral AADC inhibitor. Peripheral AADC inhibitors such as carbidopa or benserazide prevent peripheral decarboxylation of L-dopa and 5-HTP increasing the proportion that reaches the brain (Bartholini *et al*, 1967). This in turn allows the dosage of L-dopa and 5-HTP to be reduced. Inhibition of MAO and in some cases, COMT are also common treatments for these conditions in order to prevent the degradation of monoamines (Hyland, 2007; Pearl *et al*, 2007).

1.4.4 Related Conditions

The decarboxylation of 5-HTP and L-dopa performed by AADC requires PLP as an obligatory cofactor. The genetic condition pyridox(am)ine 5'-phosphate oxidase (PNPO) deficiency affects the enzyme that converts the B_6 vitamers pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate into the active vitamer PLP (Mills *et al*, 2005; see figure 1.5). This condition presents with PLP responsive seizures that develop in the neonatal period. PLP is also involved in many other enzymatic reactions including those involved in γ-amino butyric acid (GABA), glycine and glutamate metabolism (reviewed by Clayton, 2006). It is most likely the alterations of these neurotransmitters that lead to seizures. Serotonin and dopamine metabolism are also affected, although this may be variable between individuals (Hoffmann *et al*, 2007). PNPO deficiency is treated by direct replacement of PLP. A secondary central deficiency of monoamines can also occur in phenylalanine hydroxylase deficiency, where accumulated blood L-phenylalanine prevents the transport of the monoamine precursors L-tryptophan and L-tyrosine into the brain (Surtees and Blau, 2000; Bonafe *et al*, 2001b). An inherited disorder affecting the breakdown of serotonin and dopamine has also been described (Brunner *et al*, 1993a; Brunner *et al*, 1993b). This condition affects an isoform of monoamine oxidase, termed MAO-A and is associated with mild mental retardation and aggressive behaviour. 24 hour urine analysis revealed increased levels of MAO substrates including serotonin. Recently a genetic condition affecting the

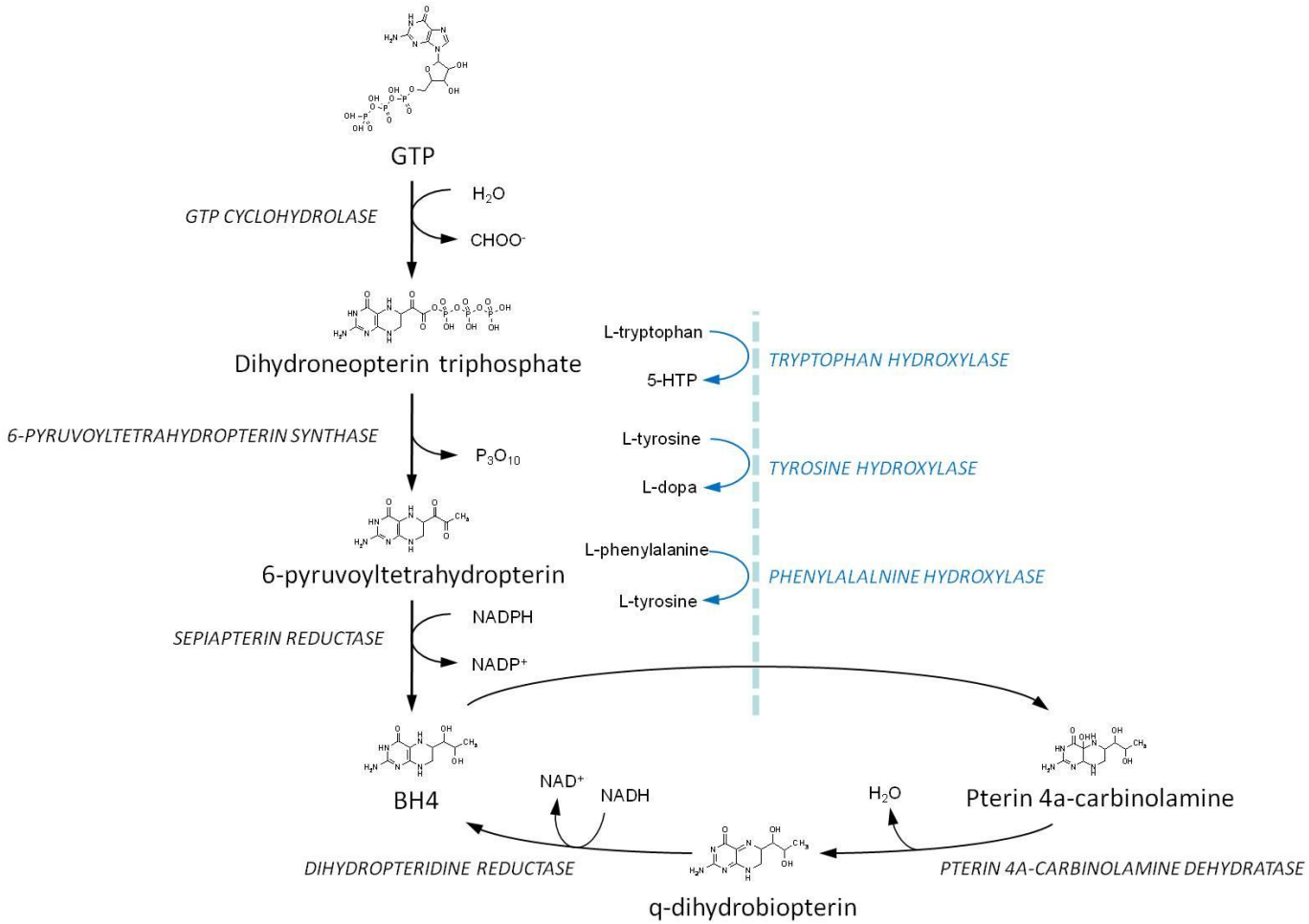


Figure 1.4 Tetrahydrobiopterin metabolic pathway. GTP: Guanosine triphosphate, BH_4: tetrahydrobiopterin, L-dopa: L-3,4-dihydroxyphenylalanine, 5-HTP:L-5-hydroxytryptophan.

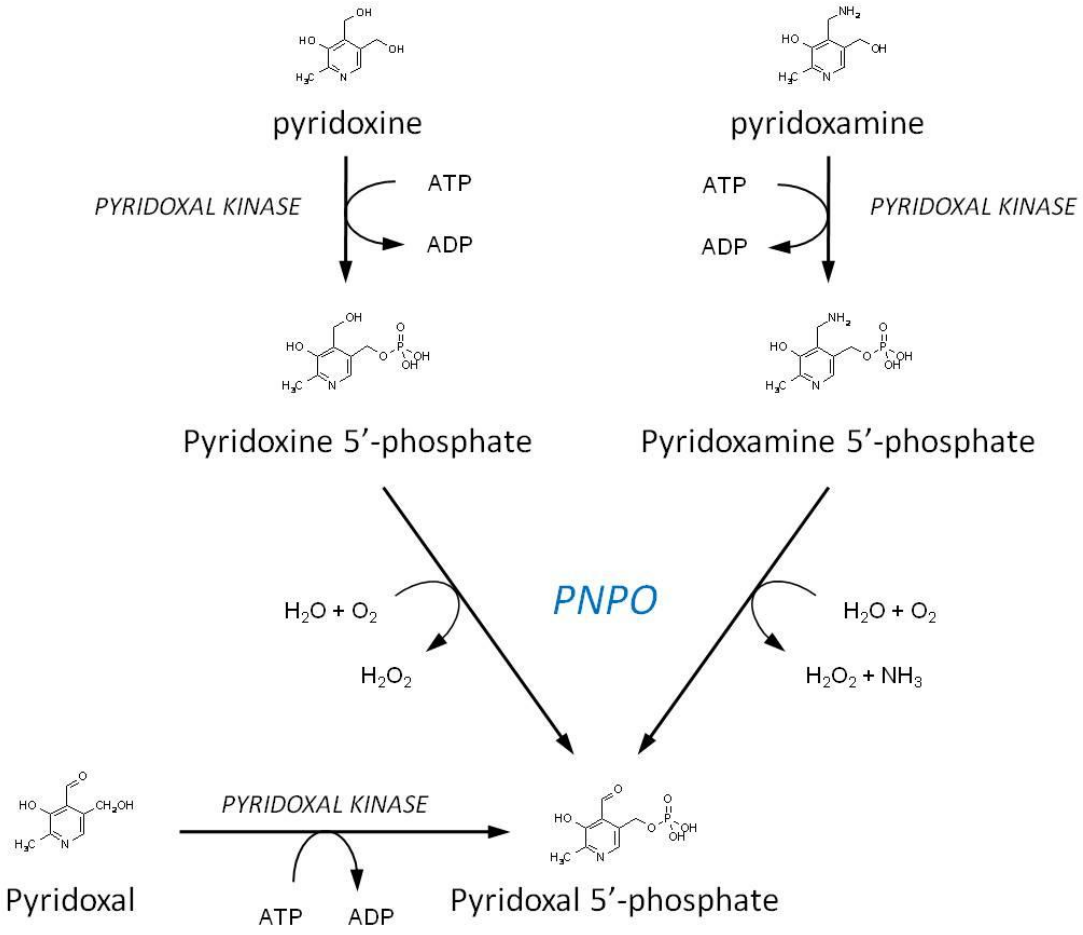


Figure 1.5 Pyridoxal 5'-phosphate metabolic pathway. ATP: adenosine triphosphate, ADP: adenosine diphosphate. PNPO: pyridoxamine 5'-phosphate oxidase.

re-uptake of dopamine via the dopamine transporter has also been described (Kurian *et al*, 2009). These patients presented with dystonia and parkinsonism and were found to have increased levels of the dopamine metabolite homovanillic acid in cerebrospinal fluid (CSF). DAT deficiency patients were not responsive to L-dopa therapy and instead dopamine agonists were suggested to be a potential treatment.

1.5 AADC deficiency

1.5.1 Clinical Picture

AADC deficiency was first diagnosed in male monozygotic twins who presented at 2 months of age with hypotonia, developmental delay, oculogyric crises and choreoathetoid limb movements (Hyland and Clayton, 1990; Hyland *et al*, 1992). Since then cases of AADC deficiency have been identified in Europe, Asia, North America and Australia and there are currently 78 cases recorded on the international patient database ((http://www.biopku.org/BioPKU_databasesJAKE.asp) curated by N. Blau). Hypotonia and oculogyric crises are the two most commonly reported symptoms of AADC deficiency (Brun *et al*, 2010). In addition other common symptoms include developmental delay, hypokinesia, choreoathetosis, dystonia, limb hypertonia, insomnia, irritability, feeding and speech difficulties (Hyland *et al*, 1992; Swoboda *et al*, 1999; Swoboda *et al*, 2003; Pons *et al*, 2004; Brun *et al*, 2010). Autonomic symptoms are also common, including nasal congestion, temperature instability, ptosis, excessive sweating and hypersalivation. Hypoglycaemia has been reported in some cases and this could also be related to autonomic deficits as noradrenaline and adrenaline can play a role in modulation of glucose metabolism (Nonogaki, 2000; Swoboda *et al*, 2003). Furthermore patients with dopamine β-hydroxylase deficiency have been reported to suffer from episodes of hypoglycaemia (Robertson *et al*, 1991), again indicating the role of the autonomic system in this symptom. Seizures have occasionally been reported in AADC deficiency and one case was responsive to antiepileptic medication (Swoboda *et al*, 2003; Hsieh *et al*, 2005; Anselm and Darras, 2006; Ito *et al*, 2008). Long-term electroencephalogram of one AADC deficient patient has demonstrated activity corresponding to epileptic seizures during involuntary movements (Ito *et al*, 2008). It is unknown if involuntary movements in AADC deficiency are commonly associated with epilepsy.

1.5.2 Diagnosis

Once AADC deficiency is suspected from the clinical presentation confirmation of the diagnosis requires biochemical analysis of CSF, measurement of plasma AADC enzyme activity and finally identification of the mutation (Hyland and Clayton, 1992). Abnormalities in dopamine and serotonin metabolism are detected by measurement of the concentrations of the major dopamine metabolite HVA, the major serotonin metabolite 5-HIAA and 3-O-methyldopa (3-OMD; 3-methoxytyrosine) a major L-dopa metabolite using high performance liquid chromatography (HPLC) coupled to electrochemical detection (ECD; Hyland and Clayton; 1992; Hyland, 2008). Due to the deficit of dopamine and serotonin in AADC deficiency both HVA and 5-HIAA are greatly reduced in patient CSF (Patient range, HVA: <2-128 nM, 5-HIAA: <2-53 nM; Reference Range, HVA: 320-955 nM, 5-HIAA: 120-480nM; Hyland and Clayton, 1992; Fiumara *et al*, 2002; Brun *et al*, 2010). 3-OMD is usually elevated in AADC deficiency due to the metabolic block at AADC (Patient Range: 54-4293 nM, Reference Range: <100nM; Hyland *et al*, 1992; Maller *et al*, 1997; Brun *et al*, 2010). 5-HTP accumulation in CSF of AADC deficient patients is also common (Patient Range: 51-264 nM; Reference Range: <10nM; Abeling *et al*, 1998; Maller *et al*, 1997; Brun *et al*, 2010). This CSF metabolite pattern is indicative of AADC deficiency however a similar pattern has been found in several patients with PNPO deficiency (Mills *et al*, 2005; Hoffmann *et al*, 2007). Measurement of AADC activity in plasma can be used to confirm the diagnosis. Plasma is incubated sequentially with PLP and L-dopa and the amount of dopamine produced is quantified by HPLC-ECD. Plasma AADC activity is very low in patients with AADC deficiency (Patient Range: <1-5 pmol/min/mL, Reference Range 36-129pmol/min/mL; Hyland and Clayton, 1992; Brun *et al*, 2010). Finally, molecular analysis of the AADC gene is generally performed in order to determine the pathogenic mutations.

1.5.3 Inheritance

AADC deficiency follows an autosomal recessive pattern of inheritance and affected individuals have been reported to be homozygous or compound heterozygous (Chang *et al*, 1998; Tay *et al*, 2007; Lee *et al*, 2008a). The human AADC gene is made up of 15 exons and 14 introns and is mapped to p12.1-p12.3 of chromosome 7 and encodes a 480 amino acid peptide (Ichinose *et al*, 1989; Sumi-Ichinose *et al*, 1992; Nasrin *et al*, 1992). Across the AADC deficient population mutations are heterogeneous, with a high

number of novel mutations (Chang *et al*, 1998; Tay *et al*, 2007; Haavik *et al*, 2008). Mutations have been identified across the majority of exons, with clusters of mutations centring on exons 2, 3 and 14 (see Brun *et al*, 2010). The most commonly reported mutation is the intronic ISV6+4A>T mutation found mainly in the Taiwanese population and in individuals with Chinese ancestry (Tay *et al*, 2007; Lee *et al*, 2008a; Lee *et al*, 2008b). Likewise the highest number of cases of AADC deficiency reported has been in Taiwan, where at least twelve individuals have been detected (Lee *et al*, 2008b). Of these cases a high proportion are homozygous for the ISV6+4A>T mutation which may lead to the production of a truncated protein and all of these cases had a severe phenotype (Lee *et al*, 2008a; Lee *et al*, 2008b). This mutation has also been reported in two siblings who were compound heterozygous and had a mild disease presentation (Tay *et al*, 2007). This indicates that in these cases condition severity could be directly dependent on the underlying genetic defects. This is also evident in the parents of the two mild siblings, the mother was heterozygous for the ISV6+4A>T mutation, while the father was heterozygous for a 853C>T mutation. The father's AADC activity (11.9 pmol/min/mL) was approximately 50% below the reference range while the mother's activity (5.3 pmol/min/mL) was approximately 75% below the reference range (Adult reference range: 24-43 pmol/min/mL; Tay *et al*, 2007). Heterozygous relatives of AADC deficient children with other mutations have been reported to have AADC activity levels as low as 87% below the reference range (Verbeek *et al*, 2007). Family pedigrees from three patients, constructed on an anecdotal basis, indicate a relatively high incidence of psychiatric disorders in relatives of these AADC patients (Swoboda *et al*, 2003). This could suggest that heterozygosity may influence or predispose some individuals to psychiatric conditions, which in turn may relate to reduced levels of AADC activity. However the role of heterozygous status in psychiatric disorders has not yet been systematically investigated.

1.5.4 Treatment

The first two indentified AADC deficient patients responded well to treatment with the MAO inhibitor tranylcypromine, the dopamine agonist bromocriptine and pyridoxine, a precursor of PLP (Hyland *et al*, 1992). After six months to one year of treatment in this manner both twins could perform voluntary movement, were learning to speak, were less irritable and oculogyric crises had ceased. The core treatments for AADC deficiency remain the same as those used in the first two cases, a MAO inhibitor, a

dopamine agonist and pyridoxine (Swoboda *et al*, 2003; Pons *et al*, 2004; Brun *et al*, 2010). However in a survey of 78 patients only approximately 19% were found to respond positively to treatment (Brun *et al*, 2010). Dopamine agonists are administered in AADC deficiency in an attempt to stimulate dopamine receptors and consequently replace dopamine neurotransmission. Dopamine agonists that have been administered to patients with AADC deficiency include bromocriptine, pergolide, pramipexole and ropinirole, each of which has a differential binding affinity for the different dopamine receptors (see section 1.3.3). However all have greater affinity for D2-like receptors rather than D1-like receptors, the efficacy of these drugs for movement disorders is considered to be due to action at D_2 and D_3 receptors (Kvernmo *et al*, 2006). In those patients that do respond to dopamine agonist treatment the main improvements are generally related to voluntary movement (Swoboda *et al*, 2003; Pons *et al*, 2004). MAO inhibitors (MAOIs) including tranylcypromine, selegiline and moclobemide are given to prevent the breakdown of the limited amounts of dopamine and serotonin produced by AADC deficient patients. Again response is variable but MAOIs have, in a few cases, been reported to decrease oculogyric crises duration or frequency (Korenke *et al*, 1997; Swoboda *et al*, 2003; Lee *et al*, 2008a). Administered pyridoxine is phosphorylated by pyridoxal kinase and then subsequently converted to PLP by PNPO (see figure 1.5; reviewed by Clayton, 2006). The aim of this treatment is to provide an excess of PLP, the coenzyme for AADC, which may in turn bolster or enhance residual AADC enzyme activity. Although improvements in the biochemical picture have been noted in some patients in response to pyridoxine treatment only rarely has any change been observed in the clinical phenotype (Hyland *et al*, 1992; Korenke *et al*, 1997; Maller *et al*, 1997; Swoboda *et al*, 1999; Pons *et al*, 2004).

A range of other pharmacological interventions have been attempted in patients with AADC deficiency. Several attempts have been made to specifically target the serotonin system. Administration of Zolmitriptan, a 5-HT_{1B} and 5-HT_{1D} selective agonist that is commonly used as a treatment for migraine, led to improvement in the mood of one patient (Swoboda *et al*, 2003). Buspirone which is a 5-HT_{1A} receptor agonist but also a partial D_2 receptor antagonist improved mood and limb rigidity in two patients however this treatment was curtailed after tardive dyskinesia developed as a side effect (Swoboda *et al*, 1999). Fluoxetine and paroxetine are inhibitors of serotonin re-uptake into neurons. This form of treatment led to improvement in oculogyric crises in one patient

but induced dystonia in another (Swoboda *et al*, 2003; Manegold *et al*, 2009). L-dopa therapy has been successful in three siblings with AADC deficiency (Chang *et al*, 2004; Manegold *et al*, 2009). Expression of the mutant AADC protein from these patients revealed that the mutant had a higher K_m for L-dopa than control, indicating a decreased affinity for L-dopa (Chang *et al*, 2004). L-dopa therapy has been trialled in several other patients but without any positive clinical response (Manegold *et al*, 2009). The hormone melatonin, which is produced from serotonin, is involved in the control of circadian rhythm (reviewed by Zawilska *et al*, 2009) and due to serotonin deficiency melatonin is also likely to be deficient in AADC deficiency. Sleep disturbances have been at least partially corrected in two patients following melatonin administration (Pons *et al*, 2004). Autonomic symptoms have also been treated with some success. Oxymetazoline nasal spray has been reported to be beneficial to one patient at reducing nasal congestion and trihexyphenidyl an anticholinergic drug has reduced excessive sweating in several cases (Pons *et al*, 2004).

1.6 Aromatic L-amino acid decarboxylase

1.6.1 Structure and Reaction Mechanism

The AADC enzyme is a homodimer consisting of two 480 amino acid monomers each with a predicted molecular weight of 54kDa (Ichinose *et al*, 1989; Nasrin *et al*, 1992). From analysis of the crystal structure each monomer consists of three domains; a large central domain of one β-sheet and eight α-helices; a c-terminal small domain of one β-sheet and three α-helices; and an N-terminal domain containing two α-helices (Burkhard *et al*, 2001). The homodimer binds two molecules of PLP, one molecule within the large domain of each monomer (Ishii *et al*, 1996; Burkhard *et al*, 2001). The active site is located close to the monomer-monomer interface and is composed of residues mostly of one monomer but with residues iosleucine 101 and phenylalanine 103 contributed from the opposing monomer (Burkhard *et al*, 2001). The active site contains lysine residue 303 which, in common with active site lysine residues of other PLP-dependent enzymes, forms the internal aldimine with PLP through a Schiff base linkage. This PLP-lysine linkage undergoes a transaldimination reaction with the substrate forming a Schiff base between PLP and the substrate, the external aldimine, leaving the amino group of the lysine residue as a free base. Site directed mutagenesis of lysine 303 demonstrates that this residue has further roles in the catalytic mechanism of AADC

including involvement in the hydrolysis reaction that separates PLP from the decarboxylation product (Bertoldi and Voltattorni, 2009).

To allow decarboxylation to occur in α-decarboxylases such as AADC it is predicted that the carboxyl group must lie perpendicular to the plane of the PLP ring (Dunathan, 1966). Residues histidine 192, aspartate 271, asparagine 300 and histidine 302 of AADC are all predicted to form hydrogen bonds with the external aldimine, maintaining its orientation within the active site (Bertoldi *et al*, 2001; Burkhard *et al*, 2001). Mutation of each of these residues individually produces proteins with greatly reduced decarboxylase activity that can also catalyse a decarboxylation dependent transamination producing an aldehyde, PMP and CO_2 (Bertoldi *et al*, 2001). This latter reaction can occur in wildtype AADC but only in anaerobic conditions, whereas in the mutated proteins it occurs both in the presence and absence of O_2 (Bertoldi and Voltattorni, 2000; Bertoldi *et al*, 2001). This demonstrates that altering residues involved in substrate-PLP binding can change the reaction specificity of AADC, possibly through alteration of the orientation of the substrate-PLP complex. Whilst none of the above residues have been reported to be altered in AADC deficiency several disease causing mutations are positioned close to active site residues (Chang *et al*, 1998; Haarvik *et al*, 2008). It is consequently conceivable that these mutations could also alter the reaction specificity of AADC. Therefore potentially the AADC enzyme of some patients could catalyse aberrant reactions such as the conversion of PLP to PMP, which in turn could lead to PLP deficiency in some tissues.

The structure of AADC contains a flexible loop region consisting of amino acids 328-339 (Burkhard *et al*, 2001). This region is susceptible to proteolytic cleavage *in vitro* however cleavage is prevented in the presence of PLP and a substrate analog, indicating that a conformational change occurs on substrate binding (Ishii *et al*, 1998; Matsuda *et al*, 2004). Analogous to structurally similar regions in related enzymes the alteration in the conformation of this flexible region may act to enclose the active site (Burkhard *et al*, 2001). Cleavage of this flexible region produces a protein with greatly reduced decarboxylase activity (Ishii *et al*, 1998). Furthermore the absence of the flexible structure led to a large increase in the formation of Pictet-Spengler adducts between the substrate and PLP (Bertoldi *et al*, 1999). This could indicate the necessity of enclosing the active site during catalysis to allow decarboxylation to proceed to completion.

1.6.2 Alternative Reactions

In addition to decarboxylation wildtype AADC has been demonstrated to catalyse several other reactions *in vitro*. In aerobic conditions alongside decarboxylation there is also the formation of Pictet-Spengler adducts between L-dopa or 5-HTP and PLP (Bertoldi and Voltattorni, 2000). This reaction leads to the removal of the PLP coenzyme from the active site and consequently inactivates the protein. Under anaerobic conditions a deamination reaction occurs subsequent to decarboxylation producing 3,4-dihydroxyphenylacetaldehyde and pyridoxamine 5'-phosphate (Oleary and Baughn, 1977; Bertoldi and Voltattorni, 2000). The release of CO_2 from the active site following decarboxylation is hypothesised to be a prerequisite for the protonation of the amine α-carbon. It has been suggested that O_2 acts to positively influence the release of CO_2 and consequently alters the fate of the reaction intermediate (Bertoldi and Voltattorni, 2000). AADC has also been demonstrated *in vitro* to directly deaminate L-aromatic amines including serotonin, producing ammonia and an aromatic aldehyde, which in the case of serotonin was 5-hydroxyindoleactealdehyde (Bertoldi *et al*, 1996). Alongside the serotonin reaction with AADC there was also found to be an irreversible inactivation of the enzyme through the permanent incorporation of serotonin and PLP into the structure of AADC. The physiological relevance of these side reactions is unknown however enhancement of side reactions has been demonstrated in several artificially generated AADC mutants (Bertoldi *et al*, 2001). This could suggest that some AADC deficiency pathogenic mutations could also alter the reaction specificity and side reactions of AADC. Biochemical characterisation of only one AADC deficiency causing mutated protein has been reported (Chang *et al*, 2004). This AADC mutant had an increased K_m for L-dopa but no alternative reactions were investigated. Characterisation of further mutant proteins could elucidate if alternative reactions do occur in AADC deficiency and whether this could have pathogenic implications.

1.6.3 Alternative Substrates

The name aromatic L-amino acid decarboxylase was originally suggested by Lovenberg *et al* (1962) upon their discovery that in addition to L-dopa and 5-HTP AADC was also able to decarboxylate all L-aromatic amino acids including L-tryptophan, L-*p*-tyrosine and L-phenylalanine. The reported K_m apparent of AADC ranges from 120μM to 620μM for L-dopa and 20μM to 490μM for 5-HTP, measured under various reaction

conditions with enzyme preparations of different purity and tissue origin (Lovenberg *et al*, 1962; Voltattorni *et al*, 1983; Boomsma *et al*, 1986; Moore *et al*, 1996; Jebai *et al*, 1997; Verbeek *et al*, 2007). The K_m apparent of AADC with L-*p*-tyrosine, the natural L-dopa precursor, was 13mM, with L-phenylalanine was 20mM and with L-tryptophan was 3mM (Lovenberg *et al*, 1962). Despite these high K_m values, in comparison to L-dopa and 5-HTP, the products of L-*p*-tyrosine, L-phenylalanine, and L-tryptophan; *p*-tyramine, 2-phenylethylamine and tryptamine respectively, are found at low concentrations in multiple brain regions and are termed the trace amines (Hess *et al*, 1959; Tallman *et al*, 1976; Suzuki and Yagi, 1976; Buck *et al*, 1977). Trace amines have a putative role in neuromodulation and a specific family of trace amine receptors has been identified (Borowsky *et al*, 2001). The physiological function most associated with central trace amines is the modulation of classical monoamine neurotransmission. 2-phenylethylamine and *p*-tyramine have been shown to enhance dopamine release, whilst tryptamine can enhance serotonin mediated inhibition of neuronal firing (Jones and Boulton, 1980; Knoll *et al*, 1996; Nakamura *et al*, 1998). Given that AADC is the sole enzyme required for the metabolism of the majority of trace amines it is likely that in AADC deficiency the trace amines are also deficient, although this possibility has not been investigated. As the trace amines are known to enhance dopamine and serotonin neurotransmission it is possible that a deficit of trace amines could have a secondary negative effect on monoamine signalling.

1.6.4 AADC Splicing

Multiple splice variants of AADC mRNA have been found in various human and animal tissues with alterations in both coding and non-coding regions. Two distinct mRNAs were discovered in pheochromocytoma and liver of both rat and human (Krieger *et al*, 1991; Ichinose *et al*, 1992). These species arise from alternative splicing of untranslated exon 1 and exon 2, and are considered to drive expression in non-neuronal and neuronal tissues respectively (Albert *et al*, 1992). However this distinction may be an oversimplification as the neuronal type has been isolated from kidney (Vassilacopoulou *et al*, 2004). Secondly an alternative splicing event within the coding region of AADC mRNA was discovered independently by two groups (O'Malley *et al*, 1995; Chang *et al*, 1996). mRNA isolated from human neuroblastoma cell lines was found to exist in two forms, $AADC_{480}$ and $AADC_{442}$. In the latter exon 3 was omitted and when expressed in COS-7 cells had no 5-HTP or L-dopa decarboxylase

activity (O'Malley *et al*, 1995). Vassilacopoulou *et al* (2004) discovered yet another species of AADC mRNA. This alternative transcript, named alt-DDC, was cloned from human placenta and compared to full-length $AADC_{480}$ mRNA is missing exons 10-15, but includes an alternative exon 10. In humans this splice variant of AADC is only found in placenta and kidney (Vassilacopoulou *et al*, 2004). The functionality of alt-DDC was not determined. This would be particularly interesting with regard to AADC deficiency given the multiple reports of hyperdopaminuria in AADC deficient patients (see section 1.6.5; Abeling *et al*, 1998, Abeling *et al*, 2000, Fiumara *et al*, 2002 and Abdenur *et al*, 2006; Wassenberg *et al*, 2010).

1.6.5 AADC Localisation

AADC within the brain is localised within both serotonergic and catecholaminergic neurons (Hokfelt *et al*, 1973). In the mammalian brain the majority of serotonergic neuronal cell bodies are found within the raphe nuclei and the brainstem reticular formation (reviewed by Baumgarten and Grozdanovic, 1997; Hornung, 2003). The raphe nuclei are located on the midline of the brainstem and are generally classified into two main groups, the rostral and caudal (Taber *et al*, 1960; Dahlstrom and Fuxe, 1964). Neuronal pathways from the caudal group project mostly to the brainstem and spinal cord whilst those of the rostral group mainly ascend to the forebrain (Bjorklund and Skagerberg, 1982). Ascending serotonergic projections target a wide range of structures including the basal ganglia, hippocampus, thalamus, hypothalamus and cortex (Azmitia and Segel, 1978). Spinal serotonergic pathways innervate across the grey matter especially laminae I and II of the dorsal horn and have been demonstrated to directly modulate motor and sensory neuronal activity (Basbaum and Fields, 1979; Proudfit and Hammond, 1981; Bjorklund and Skagerberg, 1982; Heckman *et al*, 2005). Central dopaminergic neurons project from the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) of the midbrain (Dahlstrom and Fuxe, 1964; Fuxe, 1965; Fuxe *et al*, 1970). The dopamine SNc neurons project to the dorsal striatum (caudate putuman) forming part of the basal ganglia motor circuit and it is this pathway that degenerates during Parkinson's disease (Hassler, 1938; Hornykiewicz, 1963; Dahlstrom and Fuxe, 1964; Fuxe, 1965). Two dopaminergic pathways originate from the VTA, termed the mesolimbic and mesocortical pathways. The mesolimbic pathway synapses predominantly in the nucleus accumbens and olfactory tubercle, but also to the lateral septum, hippocampus and amygdala (Haber, 2002). This pathway is particularly

associated with reward based learning and is implicated as a common pathway in addiction (Ikemoto, 2007). The mesocortical dopaminergic pathway projects to the prefrontal cortex and is thought to have a modulatory role in higher functions such as working memory, and plays an important role in schizophrenia (Tzschentke, 2001; Seamans and Yang, 2004; Toda and Abi-Dargham, 2007; Stone *et al*, 2007a).

AADC is also located in central noradrenergic neurons. The cell bodies of noradrenergic neurons are grouped in the pons, largely in the locus coeruleus, and in the medulla, mostly in the reticular formation (Fuxe, 1965; Hillarp *et al*, 1966). Projections originating from these cell bodies terminate in a wide range of brain areas including the spinal cord, hippocampus, cortex and amygdala. In addition to monoaminergic neurons AADC is also expressed in populations of non-monoaminergic neurons, termed D-cells (Jaeger *et al*, 1983; Jaeger *et al*, 1984; Kitahama *et al*, 1990; Eaton *et al*, 1993; Kitahama *et al*, 2009). In humans D-cells are detectable in the hypothalamus, striatum, forebrain and cortex (Kitahama *et al*, 2009). The function of D-cells is unknown, however it has been postulated that these cells could be involved in trace amine production (Jaeger *et al*, 1983). Alternatively it is possible that these neurons sequester extracellular substrates such as L-dopa and 5-HTP and convert them to dopamine and serotonin. Certainly it has been demonstrated that these neurons are able to decarboxylate exogenously administered L-dopa (Tison *et al*, 1991).

Peripherally AADC has a wide expression including in kidney, liver, lung, the gastrointestinal tract and plasma (Berry *et al*, 1996). AADC located in the enterochromaffin cells of the gastrointestinal tract is responsible for production of the majority of peripheral serotonin (Lauweryns and Van Ranst, 1988; Jonnakuty and Gragnoli, 2008). Serotonin in the gut is involved in the control of peristalsis and secretion, and the disruption of this function is likely to be involved in at least some of the gastrointestinal complications observed in patients with AADC deficiency (Swoboda *et al*, 2003). The enterochromaffin cells also release serotonin into circulation where it is taken up and stored in platelets and is involved in platelet aggregation and control of vascular tone (Jonnakuty and Gragnoli, 2008). The function of peripheral AADC in some other areas is not well defined. For example its function in plasma is unknown, but perhaps it could provide a secondary source of serotonin for platelets.

In the kidney AADC is located in the proximal straight and convoluted tubules of the nephron and converts circulating or filtered L-dopa into dopamine (Hayashi *et al*, 1990). Renal dopamine acts to inhibit Na^+ transport leading to an increase in Na^+ excretion (Pestana *et al*, 2001). Kidney AADC activity is partially regulated by the availability of L-dopa (Soares-Da-Silva and Fernandes, 1990). This may provide an explanation for hyperdopaminuria in some patients with AADC deficiency. L-dopa concentrations have been found to be elevated in urine and plasma of AADC deficient patients and L-dopa elevation in urine was accompanied by hyperdopaminuria (Abeling *et al*, 1998; Abeling *et al*, 2000; Swoboda *et al*, 1999). As low levels of HVA and 5-HIAA are detectable in the CSF of AADC deficient patients it is likely that they retain some albeit low levels of AADC activity (Pons *et al*, 2004). So although AADC activity is low, the high levels of L-dopa reaching the kidney are likely to lead to increased production of dopamine, which in some cases may lead to hyperdopaminuria. An alternative hypothesis could involve the splice variant of AADC termed alt-dopa decarboxylase (alt-DDC) that is only found in kidney and placenta (see section 1.6.4; Vassilacopoulou *et al*, 2004). Alt-DDC is missing exons 10-15, but includes an alternative exon 10. If alt-DDC is translated into a functional protein it is likely to be markedly different to the full-length AADC termed $AADC_{480}$. It is possible that alt-DDC could retain L-dopa decarboxylase activity however the functional properties of alt-DDC, including L-dopa decarboxylase activity, have not been determined. AADC has been purified from post-mortem human kidney through sequential chromatography steps (Mappouras *et al*, 1990). At each stage fractions of the eluate were collected and all those containing L-dopa-decarboxylase activity were pooled and further purified. At the end of purification a single approximately 50kDa band was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This indicates that in human kidney L-dopa-decarboxylation is performed by a single protein and that this protein is approximately 50kDa, the same predicted molecular weight as $AADC_{480}$. Alt-DDC would have a lower molecular weight and would appear on SDS-PAGE as a separate band. This evidence therefore suggests that full-length $AADC_{480}$ alone is responsible for L-dopa-decarboxylation in kidney and that alt-DDC is not involved.

1.6.6 AADC Regulation

The first indication that AADC may be a regulated enzyme came from studies of the rat

retina where L-dopa decarboxylation was found to increase in response to light (Hadjiconstantinou *et al*, 1988; Rossetti *et al*, 1989; Rossetti *et al*, 1990). The mechanism for this response was shown to be dependent on D_1-dopamine receptors and β-adrenergic receptors. Subsequent investigations of AADC regulation focused on the striatum where it was found that D_2 dopamine receptor agonists caused a decrease in L-dopa decarboxylation activity (Zhu *et al*, 1992; Hadjiconstantinou *et al*, 1993; Cho *et al*, 1999). Conversely D_1 and D_2 dopamine receptor antagonists caused an increase in activity (Zhu *et al*, 1992; Hadjiconstantinou *et al*, 1993; Cho *et al*, 1999). This evidence implies that AADC activity may be under regulation of dopamine perhaps providing a negative feedback mechanism for dopamine production. Moreover Boomsma *et al* (1989) demonstrated that Parkinson's disease patients chronically treated with L-dopa and the peripheral AADC inhibitors carbidopa or benserazide had increased plasma AADC activity compared to patients treated with L-dopa alone or untreated patients. The AADC inhibitor NSD-1015 has also been demonstrated to increase AADC mRNA and protein in PC12 cells in a dose-dependent manner (Li *et al*, 1993). This evidence further suggests a feedback mechanism for AADC activity, such that low activity results in increased expression. Furthermore selegiline when administered to mice at doses that would inhibit both MAO-A and MAO-B (10mg/Kg), thereby increasing dopamine availability, also decreased striatal AADC activity (Cho *et al*, 1996). On the other hand administered selegiline also increased AADC mRNA and protein in mouse midbrain. Pharmacological agents that antagonise serotonin receptors, particularly 5-HT_{1A} and 5-HT_{2A}, have also been demonstrated to increase AADC activity in rodent brain (Neff *et al*, 2006). Taken together the above evidence suggests that negative feedback mechanisms control AADC activity and consequently monoamine metabolism in both dopaminergic and serotonergic neurons. It is notable that two of the main treatments of AADC deficiency, dopamine agonists and MAO inhibitors, have both been shown to decrease AADC activity. However it is unknown whether these modulations of AADC determined in animals with normal levels of AADC would be relevant to AADC deficient patients where AADC activity is already minimal (see Allen *et al*, 2009).

There is also some evidence that PLP, the coenzyme for AADC, could also influence the level of AADC protein and possibly expression. AADC activity, with either L-dopa or 5-HTP as substrate, was found to be reduced in rodents fed on a vitamin B_6 deficient diet or pharmacologically depleted of vitamin B_6 (Rahman *et al*, 1982; Siow and

Dakshinamurti, 1985). In both studies adding PLP to the samples prior to measuring AADC activity did not return activity to control levels. This indicates that the reduction in activity was unlikely to be due to a lack of available coenzyme during the assay but instead may be due to a reduction in active AADC enzyme. Matsuda *et al* (2004) found decreased levels of AADC activity and protein in PLP-depleted rat pheochromocytoma cells treated with the vitamin B_6 antagonist 4-deoxypyridoxine. The authors proposed that the loss of AADC protein could relate to decreased stability of the non-PLP bound form of AADC, although no evidence of decreased cellular stability of AADC was presented. In Parkinson's disease patients treated with L-dopa the addition of pyridoxine has been demonstrated to reverse L-dopa's clinical effect, however the addition of a peripheral AADC inhibitor prevents this effect of pyridoxine (Duvoisin *et al*, 1969; Klawans *et al*, 1971). The antagonism of the pyridoxine effect upon L-dopa treatment with an AADC inhibitor could suggest that peripheral decarboxylation of L-dopa by AADC was increased by pyridoxine treatment.

1.7 AADC Deficiency Pathogenesis

1.7.1 Pathogenic Mechanism

The clinical manifestation of AADC deficiency is considered to be largely the result of serotonin and dopamine deficiency, with the deficiency of noradrenaline, adrenaline and melatonin also involved (Swoboda *et al*, 2003; Brun *et al*, 2010). Certainly the majority of clinical symptoms can be linked to deficits in the neuronal and non-neuronal systems that are ordinarily reliant on these signalling molecules (Hyland *et al*, 1992; Swoboda *et al*, 2003. However the lack of response to treatment in the majority of patients is perhaps an indication that neurotransmitter deficiency is not the only pathogenic mechanism. In particular the inadequacy with which dopamine agonists replace dopamine signalling or receptor activation provides a potential indication that the brains of the majority of AADC deficient patients may have an altered reactivity toward these chemicals. There are multiple potential explanations for how this lack of responsiveness could arise. One possibility is that as a result of monoamine deficiency developmental changes have taken place in the neuronal pathways that would ordinarily be receptive to monoamine replacement therapy. An alternative could be that, whilst these neuronal pathways have developed normally, adaptive changes, such as alteration of receptor density at synapses, have taken place in such a way as to make treatment ineffective. A further possibility may be that the state of AADC deficiency is in some

way detrimental to neuronal populations and initiates a neurodegenerative process. No investigations of brain pathological specimens from AADC deficient patients have been reported and consequently it is difficult to ascertain whether developmental, adaptive or degenerative processes have taken place. Nevertheless there is some evidence that neurodegeneration could occur in AADC deficiency. Firstly there have been reports that the disease course is progressive, with some patient's symptoms deteriorating over time (Fiumara *et al*, 2002). Secondly results of magnetic resonance imaging have indicated mild brain atrophy and other potentially degenerative changes in several cases (Lee *et al*, 2009; Manegold *et al*, 2009; Brun *et al*, 2010). Thirdly using positron emission tomography and the DAT marker ^{99m}Tc-TRODAT-1 it has been shown in one case of AADC deficiency that DAT expression was reduced (Lee *et al*, 2009). This alteration may be an adaptive change to dopamine deficiency however reduced DAT expression has also been linked to the early stages of degeneration of the nigrostriatal pathway (Wullner *et al*, 2005; Lee *et al*, 2009).

1.7.2 Precursor Accumulation

A metabolic block caused by inborn errors of metabolism can commonly lead to the accumulation of precursor molecules or their metabolites. This accumulation can often contribute to the pathogenesis of the condition. For example this mechanism may underlie the pathogenesis of hyperphenylalaninemia. Here accumulated blood phenylalanine, due to either phenylalanine hydroxylase deficiency or BH_4 deficiency, competes for uptake across the blood-brain barrier through the large neutral amino acid transporter (McKean *et al*, 1968; McKean, 1972; Curtius *et al*, 1972; Surtees and Blau, 2000; van Spronsen et al, 2009). Consequently the uptake of other vital amino acids can be inhibited and the brain can become deficient in these amino acids, which include L-tyrosine and L-tryptophan. In AADC deficiency analysis of patient CSF demonstrates that there can be an accumulation of both 5-HTP (Patient Range: 51-264 nM, Reference Range: <10nM) and L-dopa (Patient Range: <2-549 nM, Reference Range <25nM; Abeling *et al*, 1998; Maller *et al*, 1997; Brun *et al*, 2010). In addition the L-dopa metabolite 3-OMD is also generally increased in the CSF of AADC deficient patients (Patient Range: 54-4293 nM, Reference Range: <100nM; Hyland *et al*, 1992; Maller *et al*, 1997; Brun *et al*, 2010). It is possible that these accumulated substrates either directly or through alternative metabolic pathways could contribute to the pathogenesis of AADC deficiency.

1.7.3 Alternative metabolic pathways for 5-HTP

Beyond indoleamine metabolism an alternative metabolic route via the kynurenine pathway has been identified for 5-HTP (see figure 1.6; Fujiwara *et al*, 1979). Through this pathway 5-HTP is initially converted to 5-hydroxyformylkynurenine by the enzyme indoleamine 2,3-dioxygenase (IDO; EC No. 1.13.11.11; Shimizu *et al*, 1978; Littlejohn *et al*, 2000) and subsequently converted by arylformamidase (E.C. 3.5.1.9) to 5-hydroxykynurenine (Tsuda *et al*, 1974). 5-hydroxykynurenine has been shown to be decarboxylated to 5-hydroxykynuramine in rat intestine and brain (Noguchi *et al*, 1970; Tsuda *et al*, 1972). The enzyme responsible for this decarboxylation is not known, however the reaction is PLP dependent. Given its broad substrate specificity it is possible that AADC could catalyse this reaction. 5-hydroxykynuramine can also be produced directly from serotonin by the actions of IDO and arylformamidase. 5-hydroxykynurenine can alternatively be metabolised to 6-hydroxykynurenic acid, while 5-hydroxykynuramine can be converted to 4, 6-dihydroxyquinoline (see figure 1.6; Tsuda *et al*, 1972). The enzymes responsible for these latter stages have not been identified. Physiological and pathophysiological roles for the 5-HTP kynurenine metabolites have largely remained uninvestigated. However it has been demonstrated that 5-hydroxykynuramine can act as a serotonin agonist in arterial smooth muscle (Toda *et al*, 1974; Toda, 1975; Kitamura *et al*, 1979).

Whilst the 5-HTP-kynurenine metabolites are relatively uncharacterised the metabolites of tryptophan via the kynurenine pathway have received more attention (see figure 1.6). Three of these metabolites are known to have neuroactive or neurotoxic properties. Quinolinic acid has been demonstrated to be neurotoxic through glutamate N-methyl D-aspartate (NMDA) receptor activation leading to excitotoxicity and free radical production (Foster *et al*, 1983; Garthwaite and Garthwaite, 1987; Santamaria *et al*, 2001). Conversely kynurenic acid is an NMDA receptor antagonist and may protect against neuronal cell death (Lehmann, 1987; Stone *et al*, 2007b; Lee *et al*, 2008c). 3-hydroxykynurenine is an intermediate of tryptophan-kynurenine metabolism which can undergo oxidation leading to neurotoxicity through the generation of reactive oxygen species and hydrogen peroxide (Okuda *et al*, 1998; Guidetti and Schwarcz, 1999). Metabolites of tryptophan via the kynurenine pathway have been associated with disorders such as Huntington's disease, Alzheimer's disease and many infective and

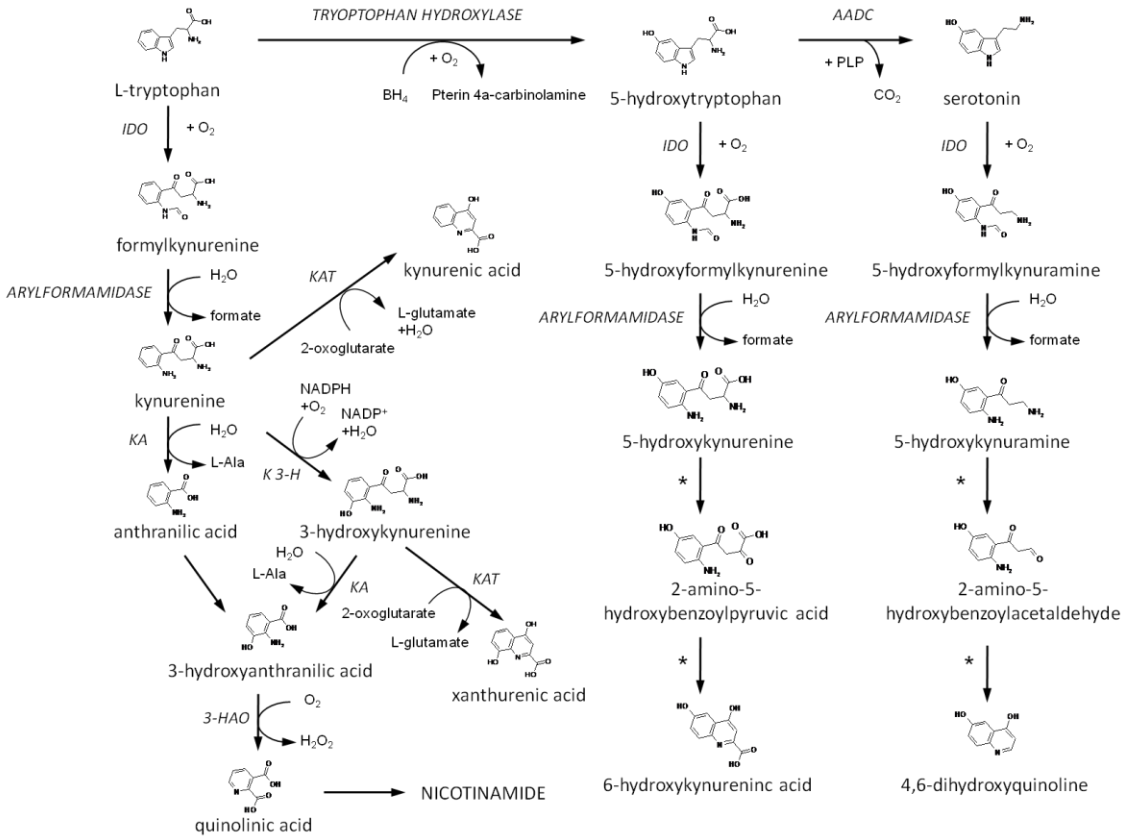


Figure 1.6 Kynurenine metabolic pathway. IDO: indolamine 2,3-dioxygenase, TPH: tryptophan hydroxylase, AADC: aromatic L-amino acid decarboxylase, KA: kynureninase, K 3-H: kynurenine 3-hydroxylase, KAT: kynurenine aminotransferase, 3-HAO: 3-hydroxyanthranilic acid oxidase, AADC: aromatic L-amino acid decarboxylase, PLP: pyridoxal 5-phosphate, L-Ala: L-alanine, BH_4: tetrahydrobiopterin, *unknown enzyme(s).

inflammatory conditions (Bruyn and Stoof, 1990; Guillemin *et al*, 2003; Guillemin *et al*, 2005a; Leonard, 2007). It is possible that 5-HTP-kynurenine metabolites could have similar neurotoxic properties to tryptophan-kynurenine metabolites, although this possibility has not been investigated. For example 5-hydroxykynurenine differs from 3-hydroxykynurenine only in the position of the hydroxyl group and conceivably could also be susceptible to oxidation. The accumulation of 5-HTP in AADC deficiency could lead to an increase in the levels of 5-HTP-kynurenine metabolites and consequently may contribute to the pathogenesis of this disorder.

1.7.4 Alternative Metabolic Pathways for L-dopa

The main alternative metabolic pathway to catecholamine metabolism for L-dopa is initiated by COMT which methylates L-dopa to 3-OMD with SAM acting as a methyl donor (see figure 1.7; Alexrod and Tomchick, 1958; Pletscher *et al*, 1967; Fellman *et al*, 1971). 3-OMD is subsequently converted to vanilpyruvic acid in a reaction catalysed by tyrosine aminotransferase, requiring 2-oxoglutarate as an amino group acceptor and pyridoxal 5'-phosphate as a co-enzyme (Miller and Litwack, 1971; Maeda *et al*, 1978). Vanilpyruvic acid is reduced to vanillactic acid (VLA) by aromatic α-ketoacid reductase, although this reaction can also be catalysed by lactate dehydrogenase, both reactions require reduced nicotinamide adenine dinucleotide (NADH) as an electron donor (Zannoni and Weber, 1966; Meada *et al*, 1978). Tyrosine aminotransferase and α-ketoacid reductase have been detected in human liver, heart, kidney and brain (Fellman *et al*, 1969; Donald, 1982). VLA is excreted in urine, and is commonly found in high levels in the urine of patients with AADC deficiency (Goodwin *et al*, 1978; Hyland and Clayton, 1992; Abeling *et al*, 1998). Studies in rats have suggested that 3-OMD at high concentrations competes with L-dopa for transport across the blood-brain barrier, consequently reducing L-dopa accumulation and dopamine turnover (Wade and Katzman, 1975; Reches and Fahn, 1982; Lee *et al*, 2008d). However 3-OMD, at plasma concentrations replicating those found in Parkinson's disease patients treated with L-dopa (39 ± 4 μM), was not found to inhibit the uptake of 6-[^{18}F]fluorodopa in Macaques (Guttman *et al*, 1992). These plasma concentrations are higher than those found in AADC deficiency (Patient Range: 9-10.5μM; Hyland *et al*, 1992; Maller *et al*, 1997), indicating that 3-OMD is unlikely to block blood-brain barrier transport in AADC deficient patients.

L-dopa is also a substrate for the biosynthesis of the pigment melanin (reviewd by Riley, 1997; Pattison *et al*, 2002). The initial enzyme in melanin synthesis is tyrosinase, a bifunctional enzyme that catalyses the hydroxylation of tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone (Hearing and Ekel, 1976). Dopaquinone subsequently undergoes a series of spontaneous and enzymatically catalysed reactions to form the indolic polymer melanin (Riley, 1997; Pattison *et al*, 2002). The reactions of melanin formation occur within specialised membrane bound organelles termed melanosomes (Seiji and Fitzpatrick, 1961; Seiji *et al*, 1963). Tyrosinase is not expressed in the brain (Gimenez *et al*, 2003; Tribl *et al*, 2007), however L-dopa can also readily undergo auto-oxidation producing dopaquinone via a free radical intermediate, dopa-semiquinone (Monder et al, 1957a; Felix and Sealy, 1981; Pattison *et al*, 2002). The oxidation of L-dopa to dopa-semiquinone and its subsequent oxidation to dopaquinone can be catalysed by transition metal ions (Monder *et al*, 1957b). Furthermore dopa-semiquinone can react with O_2 and H_2O_2 producing $O_2^{\bullet-}$ and the $OH^{\bullet-}$ respectively (Pattison *et al*, 2002). Dopamine can undergo a similar auto-oxidation process. Auto-oxidation of dopamine, and to a lesser extent L-dopa, is thought to lead to the production of neuromelanin, a characteristic feature of dopaminergic nigrostriatal neurons (Wakamatsu *et al*, 2003). Neuromelanin is a dark pigment contained within a membrane bound autophagic vacuole. The production of neuromelanin may be driven by excess cytosolic catecholamines (Sulzer *et al*, 2000). If excess cytosolic L-dopa can drive neuromelanin production then patients with AADC deficiency, in whom L-dopa accumulates, may have increased levels of neuromelanin. L-dopa as well as dopamine have been demonstrated to be toxic *in vitro* due to auto-oxidation, leading to the production of hydrogen peroxide and melanin, the induction of apoptotic pathways and lipid peroxidation (Mena *et al*, 1993; Basma *et al*, 1995; Lai and Yu, 1997; Nakao *et al*, 1997; Offen *et al*, 2001; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003), however *in vivo* experiments indicate that L-dopa may not be toxic (Mytilineou *et al*, 2003; LeWitt and Nyholm, 2004; Muller *et al*, 2004). Furthermore L-dopa has been demonstrated to have some neurotrophic and neuroprotective effects at sub-toxic concentrations (Mytilineou *et al*, 1993; Han *et al*, 1996; Mena *et al*, 1997a; Jia *et al*, 2008a). The accumulation of L-dopa seen in AADC deficiency leads to an accumulation of 3-OMD, which can be seen in CSF of AADC patients. This excess of L-dopa may also undergo auto-oxidation with potential neurotoxic and neuroprotective effects that could influence the pathogenesis of AADC deficiency.

1.7.5 Oxidative Stress

Both 5-HTP and L-dopa accumulation has the potential to produce reactive oxygen species (ROS) either via direct oxidation or through metabolism (Monder et al, 1957a; Felix and Sealy, 1981; Ng *et al*, 2000; Pattison *et al*, 2002; Smith *et al*, 2009). ROS is a collective term for oxygen derived reactive molecules including both free radicals such as $O_2^{\bullet-}$ and non-radicals such as hydrogen peroxide (H_2O_2; Halliwell, 1992). Reactions involving ROS can lead to cellular damage such as adduct formation with DNA guanine nucleotides or lipid peroxidation (Halliwell and Gutteridge, 1984; Steenken, 1989). Oxidative stress refers to a circumstance where the generation of ROS outweighs a cells capability to detoxify ROS and repair damage generated by ROS. The central nervous system (CNS) may be particularly susceptible to oxidative stress due to its unique physiological functions (reviewed by Halliwell, 1992; Halliwell, 2006). The mammalian brain has an especially high requirement for O_2 which in turn is due to the high usage of adenosine triphosphate (ATP). For example ATP is utilised to maintain ion concentration gradients across membranes required for essential neuronal functions such as maintaining membrane potentials and neurotransmitter transport such as via SERT, DAT and VMAT2 (Dunham and Glynn, 1961; Tissari *et al*, 1969; Xie and Stone, 1986; Moriyama and Nelson, 1987; Cidon and Sihra, 1989; Skou, 1998). The vast majority of O_2 is reduced to H_2O by cytochrome c oxidase (complex IV) of the mitochondrial electron transport chain (Sas *et al*, 2007). However also at the mitochondrial electron transport chain approximately 1-2% of O_2 is reduced to $O_2^{\bullet-}$ due to electron 'leakage' mostly at complex I, but also at complex III (Kudin *et al*, 2005). $O_2^{\bullet-}$ can be reduced to H_2O_2 by manganese-containing superoxide dismutase (MnSOD) in the mitochondrial matrix or copper and zinc containing superoxide dismutase (CuZnSOD) in the rest of the cell (Okado-Matsumoto and Fridovich, 2001). H_2O_2 is also produced in the brain by MAO during the degradation of serotonin, dopamine and noradrenaline (McEwen, 1965; Spina and Cohen, 1989). Catalase and glutathione peroxidase (GPx) can detoxify H_2O_2 however both may only be present at low concentrations within some brain tissues (de Marchena *et al*, 1974; Mavelli *et al*, 1982; Halliwell, 2006; see section 1.7.6). The brain may also be more susceptible to oxidative stress due to a relatively high iron content and potentially a low iron bind capacity in the CSF, increasing the possibility of $OH^{\bullet-}$ production via the Fenton reaction (Harrison *et al*, 1968; Bleijenberg *et al*, 1971; reviewed by Gutteridge, 1994; Bradbury, 1997). Furthermore neuronal membranes are rich in polyunsaturated fatty acids increasing their

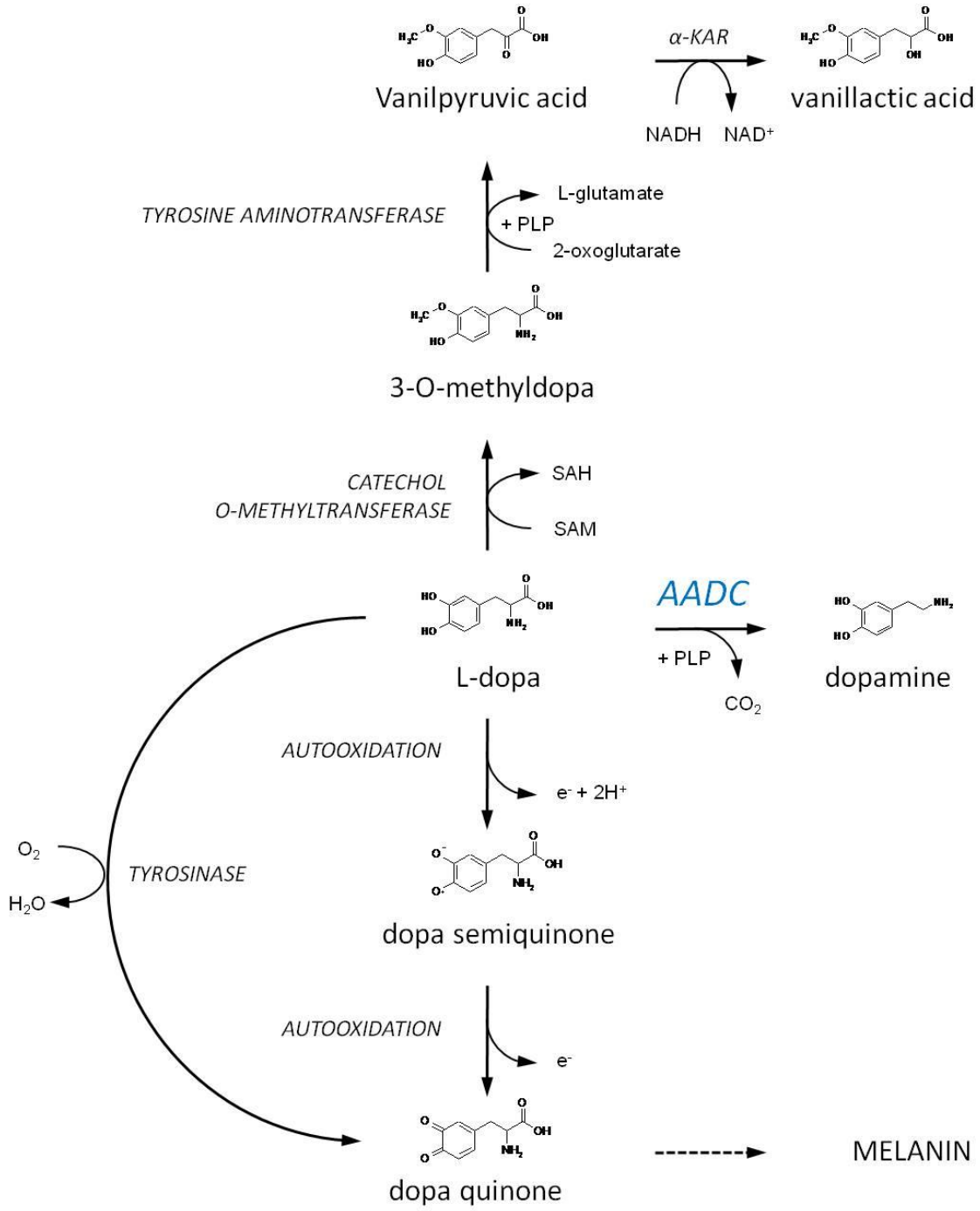


Figure 1.7 L-dopa catabolism. AADC: aromatic L-amino acid decarboxylase, PLP: pyridoxal 5'-phosphate, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, α-KAR: α-ketoacid reductase.

susceptibility to lipid peroxidation (reviewed by Farooqui and Horrocks, 1998).

1.7.6 Glutathione

One of the most important cellular defences against oxidative stress is reduced glutathione (GSH) which is the most abundant non-protein cellular thiol (reviewed by Ballatori et al, 2009). GSH is synthesised through the action of γ-glutamylcysteine ligase that forms the dipeptide γ-glutamylcysteine from glutamate and cysteine, and subsequently glutathione synthase which adds glycine to γ-glutamylcysteine to form the tripeptide GSH (see figure 1.8; Mandeles and Bloch, 1955; Snoke and Bloch, 1955). Both stages of GSH synthesis require ATP and γ-glutamylcysteine ligase is the rate limiting enzyme for the formation of GSH. The antioxidant capability of GSH is two-fold; firstly GSH can be directly oxidised by ROS to form glutathione disulphide (GSSG); secondly GSH can act as an electron donor for the conversion of H_2O_2 to $2H_2O$ by GPx, again producing GSSG (reviewed by Martin and Teismann, 2009; Ballatori *et al*, 2009). GSSG is reduced back to form two molecules of GSH by the enzyme glutathione reductase using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (Rall and Lehninger, 1952; Scott *et al*, 1963). This redox cycling between GSH and GSSG, in combination with other redox couples such as thioredoxin, allows the cell to maintain a redox balance and detoxify reactive oxygen species (reviewed by Kalinina et al, 2008).

Within the brain and in cell culture the GSH content of astroglial cells is higher than that of neurons (Slivka *et al*, 1987; Raps *et al*, 1989; Bolanos *et al*, 1995). The intracellular GSH concentration in primary neurons cultured separated from glia was found to decrease over time (Sagara *et al*, 1993). This decrease is considered to be due to the inability of neurons to utilise cystine to form reduced cysteine as a precursor of GSH (Sagara *et al*, 1993; Kranich *et al*, 1996). Conversely glial cells are able to utilise cystine in the generation of GSH (Kranich *et al*, 1998). In neuronal-glial co-cultures neuronal GSH content is maintained, demonstrating that glial cells provide a source of cysteine for neuronal GSH synthesis (see figure 1.9; Sagara *et al*, 1993; Dringen *et al*, 1999). It has been shown that astrocytes release GSH (Yudkoff *et al*, 1990; Sagara *et al*, 1996) and in addition astrocytes release superoxide dismutase (SOD) which prevents the extracellular oxidation of GSH (Stone *et al*, 1999; Stewart *et al*, 2002; Pope *et al*, 2008). Released GSH can be subsequently broken down by the enzyme γ-glutamyl transpeptidase (γ-GT), expressed on the cell surface of astrocytes, to form

cysteinylglycine (CysGly) whilst transferring the γ-glutamyl group to an acceptor (Dringen *et al*, 1997). Inhibiting γ-GT prevented the increase in neuronal GSH content seen when these cells were co-cultured with astrocytes (Dringen *et al*, 1999). It has been further demonstrated that CysGly is hydrolysed by aminopeptidase N, expressed on the surface of neurons, to give cysteine and glycine (Dringen *et al*, 2001). The action of aminopeptidase N is also required for the astrocyte-dependent increase in neuronal GSH. This release and subsequent breakdown of GSH from astrocytes may provide much of the amino acids, particularly cysteine for GSH synthesis within neurons (reviewed by Dringen and Hirrlinger, 2003). It is possible that the accumulation of L-dopa and/or 5-HTP in AADC deficiency could increase the production of ROS in the brain of AADC deficient patients. This proposed increase in ROS production could in turn disrupt GSH homeostasis potentially leading to oxidative stress.

1.7.7 Mitochondrial Respiratory Chain

The mitochondrial electron transport chain consists of four multi-subunit complexes bound to the inner mitochondrial membrane (see figure 1.10). Complex I (NADH:ubiquinone oxidoreductase) transfers two electrons from NADH via flavin mononucleotide and iron-sulfur prosthetic groups to reduce ubiquinone to ubiquinol (reviewed by Hirst, 2010). This electron transfer drives the transport of $4H^+$ across the mitochondrial inner membrane from the matrix to the intermembrane space. Complex II (succinate:ubiquinone oxidoreductase) oxidises succinate to fumarate as part of the citric acid cycle, transferring the electrons to covalently bound flavin adenine dinucleotide (FAD) to generate $FADH_2$ (reviewed by Cecchini, 2003). Electrons are then transferred from $FADH_2$ via iron-sulphur clusters to reduce ubiquinone to ubiquinol.

Complex III (ubiquinol:cytochrome c oxidoreductase) contains three heme centres termed cytochrome b_L, cytochrome b_H and cytochrome c_1 as well as two ubiquinone binding sites, Q_o that binds one molecule of reduced ubiquinol and Q_i that binds one molecule of oxidised ubiquinone (De Vries *et al*, 1982; reviewed by Crofts, 2004). One electron from bound ubiquinol is transferred via an iron sulphur cluster and cytochrome c_1 to reduce cytochrome c, a small water soluble heme- containing protein. This reduced cytochrome c can then diffuse away from complex III. The remaining electron from ubiquinol is transferred via cytochrome b_L and cytochrome b_H to the oxidised

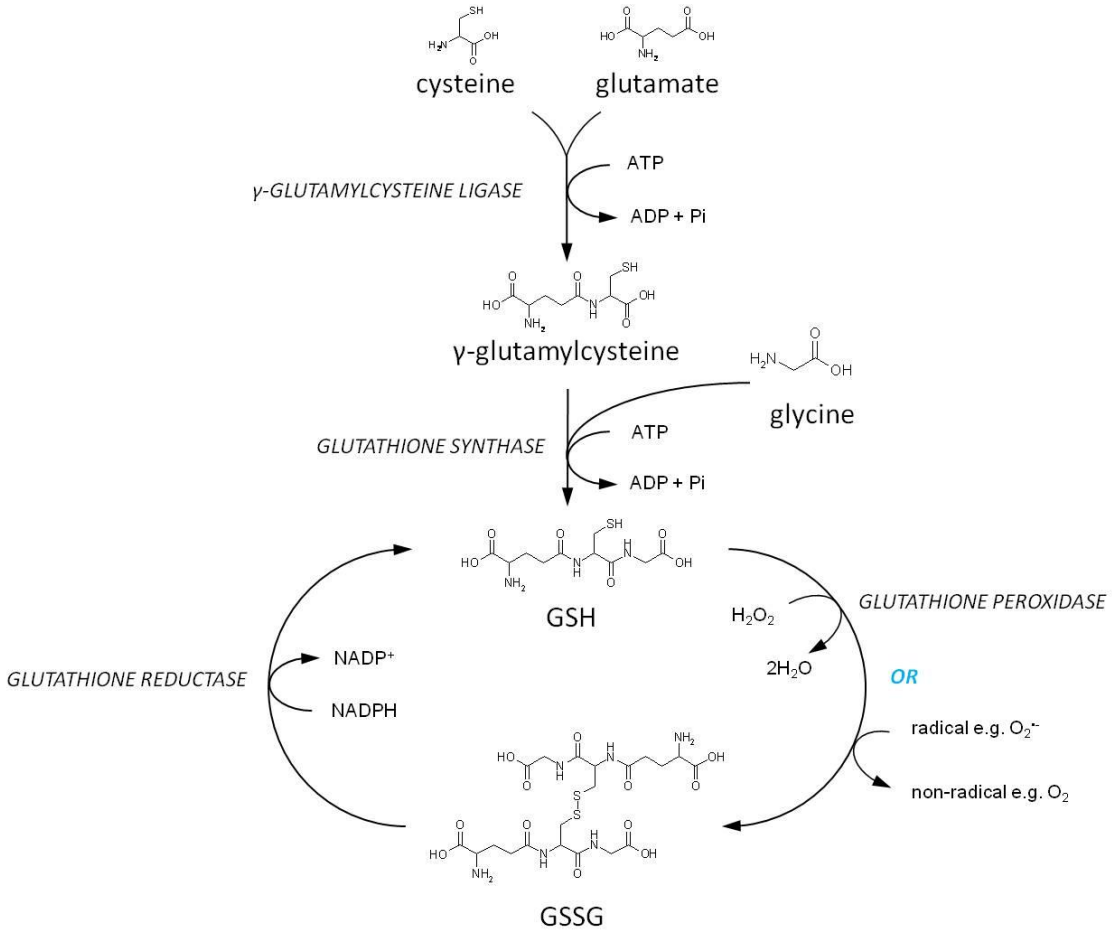


Figure 1.8 Glutathione synthesis and recycling. GSH: reduced glutathione, GSSG: glutathione disulphide.

ubiquinone bound at Q_i producing a ubisemiquinone. This process is then repeated with a second molecule of reduced ubiquinol binding at Q_o, transferring one electron to a cytochrome c molecule and a second electron to ubisemiquinone at Q_i to give ubiquinol. With each reduction of a ubiquinol molecule $2H^+$ are released to the intermembrane space. In addition $2H^+$ from the mitochondrial matrix are utilised by the reduction of ubiquinone at Q_i.

Complex IV (cytochrome c oxidase) catalyses the final step in the electron transport chain, the reduction of O_2 to $2H_2O$ using four molecules of oxidised cytochrome c (reviewed by Brzezinski and Gennis, 2008). This complex contains two copper centres (Cu_A and Cu_B) and two heme centres ($Heme_a$ and $Heme_{a3}$). An electron from the first molecule of oxidised cytochrome c is transferred via Cu_A and both heme centres to Cu_B, reducing Cu^{2+} to Cu^+. An electron from a second molecule of cytochrome c is transferred via Cu_A and $Heme_a$ to $Heme_{a3}$, reducing Fe^{3+} to Fe^{2+}. Oxygen then binds initially to $heme_{a3}$ and then additionally to Cu_B, forming a peroxide bridge between these two centres. Third and fourth electrons from two oxidised cytochrome c molecules and $2H^+$ from the mitochondrial matrix sequentially break this bond and produce hydroxyl groups bound to the copper and iron in Cu_B and $heme_{a3}$ respectively. The final addition of $2H^+$ from the mitochondrial matrix generates $2H_2O$ from the hydroxyl groups. In addition $4H^+$ are transferred from the matrix to the intermembrane space during this process (Wikstrom, 1977).

Complexes I, III and IV all contribute during electron transport to the H^+ gradient across the mitochondrial inner membrane. This H^+ gradient is used to drive the final stage in oxidative phosphorylation, ATP generation via ATP synthase (complex V). ATP synthase is a multi-subunit complex consisting of two main domains F_1 and F_0. F_0 is a transmembrane domain tranversing the mitochondrial inner membrane, whilst F_1 is soluble and positioned on the matrix side of the inner membrane (reviewed by von Ballmoos *et al*, 2008). Binding and transloction of H^+ ions at F_0 from the intermembrane space to the matrix generates rotation in the structure of the complex (Walker *et al*, 1998; Stock *et al*, 1999; Stock *et al*, 2000). This rotation changes the conformational state of subunits within the F_1 domain altering the binding affinity for adenine nucleotides and driving ATP synthesis. However the reaction is reversible and when the H^+ gradient across the mitochondrial inner membrane decreases ATP synthase can utilise ATP hydrolysis to transfer H^+ ions in the opposite direction.

The mitochondrial respiratory chain as well as being a potential source of ROS (see section 1.7.5) may also be sensitive to oxidative stress. An accumulation of substrates in AADC deficiency could hypothetically increase the production of ROS in the brain, which in turn may lead to damage to the mitochondrial respiratory chain. ROS have been demonstrated to damage all components of the respiratory chain *in vitro* and induction of oxidative stress or depletion of GSH can affect activity of the complexes *in vivo* (Zhang *et al*, 1990; Benzi *et al*, 1991; Bolanos *et al*, 1994; Heales *et al*, 1995; Bolanos *et al*, 1996; Merad-Boudia *et al*, 1998; Merad-Saidoune *et al*, 1999; Chinta and Andersen, 2006). Furthermore inhibition of complex I can in turn increase $O_2^{\bullet-}$ production potentially exacerbating oxidative stress (Lambert and Brand, 2004). Damage to the mitochondrial respiratory chain could reduce ATP synthesis, decreasing energy supply to the cell. This may be particularly detrimental to neurons as glycolysis is actively downregulated in these cells and consequently cannot compensate for the loss of oxidative phosphorylation (Almeida *et al*, 2001; Herrero-Mendez *et al*, 2009). In addition decreased respiratory chain function can lead to dissipation of the mitochondrial membrane potential, altered Ca^{2+} homeostasis, opening of the mitochondrial permeability transition pore and cell death through the release of pro-apoptotic proteins including cytochrome c into the cytoplasm (Wu *et al*, 1990; Bernardi and Petronilli, 1996; Packer et al, 1996; Chernyak and Bernardi, 1996; Clayton *et al*, 2005; Wu *et al*, 2007; Oppenheim *et al*, 2009).

1.8 Hypotheses

1) 5-HTP accumulation in AADC deficiency is toxic to neurons or glial cells either directly or via the kynurenine pathway through the induction of oxidative stress.

2) The accumulation of L-dopa in AADC deficiency leads to the induction of oxidative stress and neuronal cell death.

3) The availability of the AADC cofactor PLP influences the stability or expression of the AADC enzyme.

1.9 Aims

1) To establish the plasma AADC activity assay as a diagnostic test in the UK.

2) To investigate the effect of 5-HTP accumulation on neuronal cell viability and oxidative stress by determining cellular GSH levels and mitochondrial respiratory chain function in cell culture.

3) To investigate the potential role of L-dopa accumulation in the generation of oxidative stress by examining cell viability, GSH homeostasis and mitochondrial respiratory chain function in cell culture.

4) To investigate the role of PLP in the regulation of AADC availability.

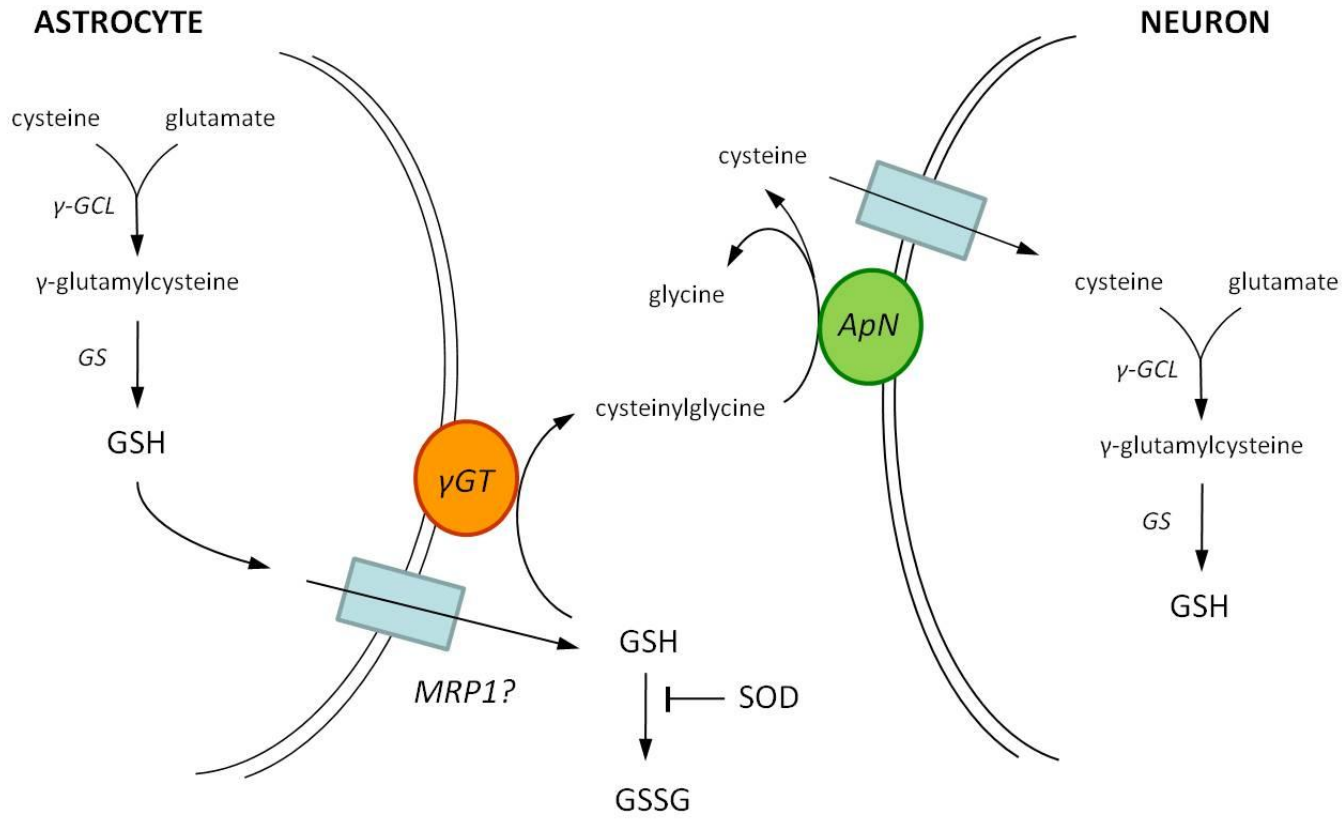


Figure 1.9 Glutathione trafficking from astrocytes to neurons. γ-GCL: γ-glutamylcysteine ligase, GS: glutathione synthase, GSH: reduced glutathione, MRP1: multidrug resistance protein 1, γ-GT: γ-glutamyltranspeptidase, ApN: aminopeptidase N, SOD: superoxide dismutase

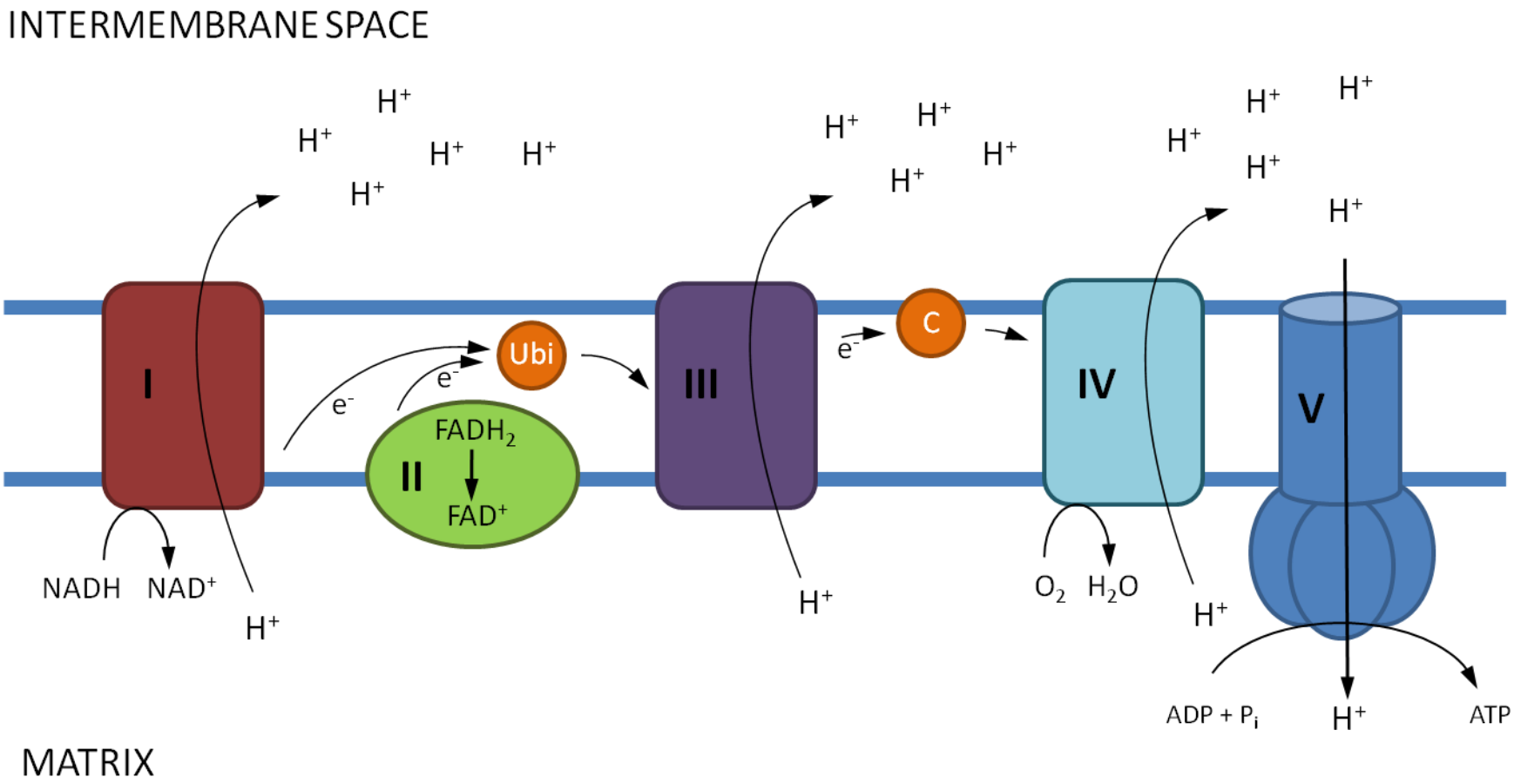


Figure 1.10 Mitochondrial respiratory chain. Ubi: ubiquinone, C: cytochrome C

Chapter 2

Material and methods

2.1 Materials

The following were purchased from VWR International Ltd (Lutterworth, UK):

Disodium hydrogen orthophosphate; sodium dihydrogen orthophosphate; potassium dihydrogen phosphate; dipotassium hydrogen phosphate; ethylenediaminetetraacetic acid (EDTA); hydrochloric acid (12.2M); methanol HiPerSolv for HPLC; potassium hydroxide; sodium hydroxide; sodium bicarbonate; sodium chloride; calcium chloride; magnesium sulfate and D-glucose.

The following were purchased from Sigma Aldrich (Poole, UK):

1,4-Dithioerythritol; pyridoxal 5'-phosphate; L-3,4-dihydroxyphenylalaine; perchloric acid; sodium-L-ascorbate; dopamine; 1-octanesulfonic acid; L-5-hydroxytryptophan; serotonin; 5-hydroxyindoleacetic acid; homovanillic acid; 3,4-dihydroxyphenylacetic acid; 3-O-methyldopa; noradrenaline; trichloroacetic acid; 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015); tranylcypromine; Ro-41-0960; SCH-23390; sulpiride; superoxide dismutase; catalase; cyclohemimide; pyridoxal; 4-deoxypyridoxine; sulforhodamine B monosodium salt; tris base; tris HCl; Sterile modified Hank's balanced salt solution with sodium bicarbonate, without phenol red, calcium chloride and magnesium sulphate (HBSS); sodium dodecyl sulphate (SDS); tween 20; triton X-100; sodium deoxycholate; mammalian protease inhibitor cocktail; sucrose; sterile 4mL/L trypan blue; dimethyl sulfoxide; fusaric acid; reduced glutathione; β-nicotinamide adeneine dinucleotide reduced; potassium cyanide; ubiquinone; cytochrome c; antimycin A; rotenone; ferricyanide; acetyl-coenzyme A; 5, 5'-dithio-bis (2-nitrobenzoic acid); oxaloacetate and sodium pyruvate.

850mL/L Orthophosphoric acid HPLC electrochemical grade was purchased from Fisher Scientific UK Ltd (Loughborough, UK).

Sterile cell culture products: Dulbecco's modified eagles medium (DMEM); DMEM/Ham's F-12 Nutrient Mixture (DMEM/F-12) (1:1); L-glutamine;; foetal bovine serum, heat inactivated; 2.5mL/L trypsin-EDTA; recombinant human interferon-γ; Nu-PAGE sample buffer; Nu-PAGE MES SDS running buffer, Nu-PAGE 4-12% Bis-Tris gel were purchased from Invitrogen Ltd (Paisley, UK).

HPLC vials and caps were purchased from Chromacol (Welwyn Garden City, UK).

Mini trans-blot filter paper and DC total protein assay kit were purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK).

Pierce ECL western blotting substrate was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

All other reagents were analytical grade and purchased from Sigma Aldrich, Fisher Scientific or VWR International except where stated.

2.2 High performance liquid chromatography

Catecholamines, indoleamines and GSH were quantified using reversed-phase high performance liquid chromatography (HPLC) coupled to electrochemical detection (ECD). Reversed-phase HPLC utilises a non-polar stationary phase and an aqueous mobile phase to separate analytes. Non-polar molecules tend to adsorb to the stationary phase and so are retained on the column longer than polar molecules (Molnar and Horvath, 1976). ECD uses an electrical potential to oxidise or reduce analytes flowing past the surface of electrodes, generating a current that can be used to quantify the analyte (reviewed by Mefford, 1981). Throughout this thesis coulometric electrochemical detection was utilised, where the total amount of analyte present is oxidised and consequently the current is directly proportional to the concentration of the analyte (Mefford, 1981; Marsden and Joseph, 1986).

2.2.1 Catecholamine HPLC

2.2.2 Equipment

HPLC equipment: PU-2080 Plus pump (Jasco (UK) Ltd., Great Dunmow, UK); AS-2057 Plus autosampler (Jasco); Coulochem III electrochemical detector and 5010 analytical cell (ESA Analytical Ltd., Aylesbury, UK) assembled as in figure 2.1. The electrochemical detector was coupled to a computer and data recorded using AZUR version 4.6 chromatography data capture and analysis software (Datalys, Saint Martin D'Heres, France). The mobile phase was degassed using a Degasys populaire inline degasser (Degasys, Japan).

2.2.2.1 Procedure

This method is based on that of Hyland and Clayton (1992) adapted here for the simultaneous determination of L-dopa, 3-OMD, dopamine, DOPAC and HVA. The mobile phase consisted of 50mM potassium phosphate (pH 3.12), 1.4mM octanesulfonic acid, 0.05mM EDTA, 170mL/L methanol in HPLC grade water. The flow rate was 1.0mL/min. Samples were thawed at +20˚C, transferred to vials and loaded into the autosampler, maintained at +4˚C. 50µL of each sample was injected and separated on a C18 reversed phase 250 x 4.6mm i.d. HiQSil C18W column (KYA Tech. Corp. Tokyo, Japan), maintained at +35˚C. Analytes were detected by coulometric electrochemical detection. The screening electrode (E1) was set to +20mV to oxidise analytes of low oxidation potential. A voltamogram was used to determine the

optimum potential for the detector electrode (E2) by measuring the peak area of 500nM L-dopa, 3-OMD, dopamine, DOPAC and HVA at electrode potentials ranging from +30mV to +520mV (see figure 2.2a). An E2 electrode potential of +450mV was chosen for determination of all five analytes. Samples were quantified against external standards of 250nM L-dopa, 3-OMD, dopamine, DOPAC and HVA in 0.4M perchloric acid (see figure 2.2b). 250nM noradrenaline was run with each standard to ensure it did not co-elute with the L-dopa peak. Noradrenaline was not quantified due to co-eluting peaks within samples. Calibration curves were performed to determine the linearity between current amplitude and standard concentration (see figure 2.3a). Linearity was demonstrated for L-dopa between 1nM and 4000nM and for 3-OMD, dopamine, DOPAC and HVA 5nM and 5000nM (r^2 = 1.000 for each analyte). An example of a sample chromatogram is presented in figure 2.3b.

2.2.2.2 Data Analysis

Catecholamines were identified and quantified using the AZUR Version 4.6 software package (Datalys) using the following equation:

Conc. (nM) = (sample peak area /external standard peak area) x calibration standard conc. (nM)

For intracellular determination of catecholamines the conc. (nM) was divided by total protein (mg/mL) and expressed as pmol/mg of protein.

2.2.3 Measurement of Indoleamines by HPLC

2.2.3.1 Equipment

The equipment used was as described for catecholamine measurement, see section 2.2.2 and figure 2.1

2.2.3.2 Procedure

This method is based on the method of Alvarez *et al* (1999) with some minor modifications. The mobile phase consisted of 50mM potassium phosphate (pH 2.6), 1mM OSA, 50µM EDTA, 170mL/L methanol in HPLC grade H_2O run at 1.3mL/min. Samples were transferred to vials and loaded into the autosampler and maintained at

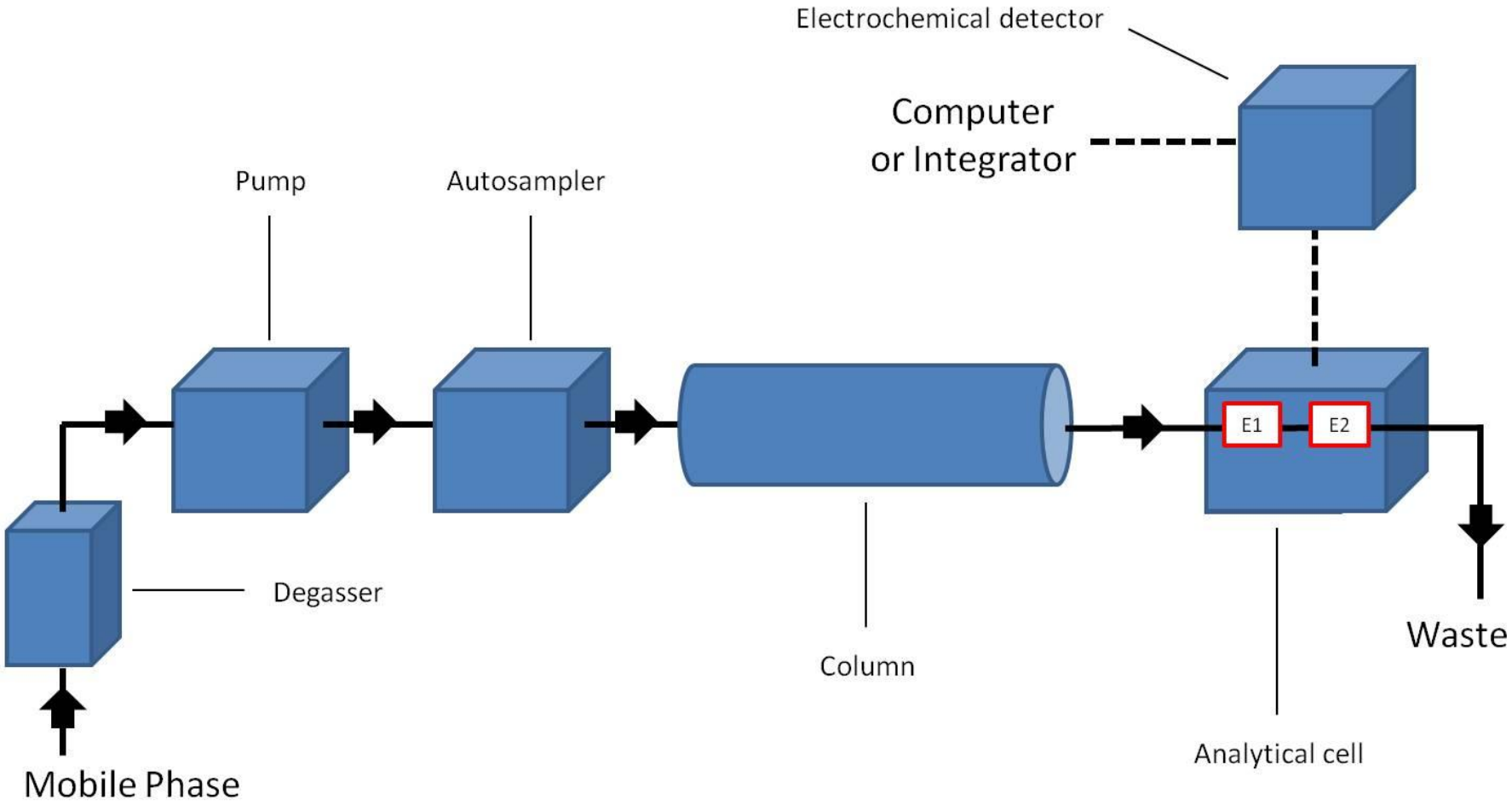
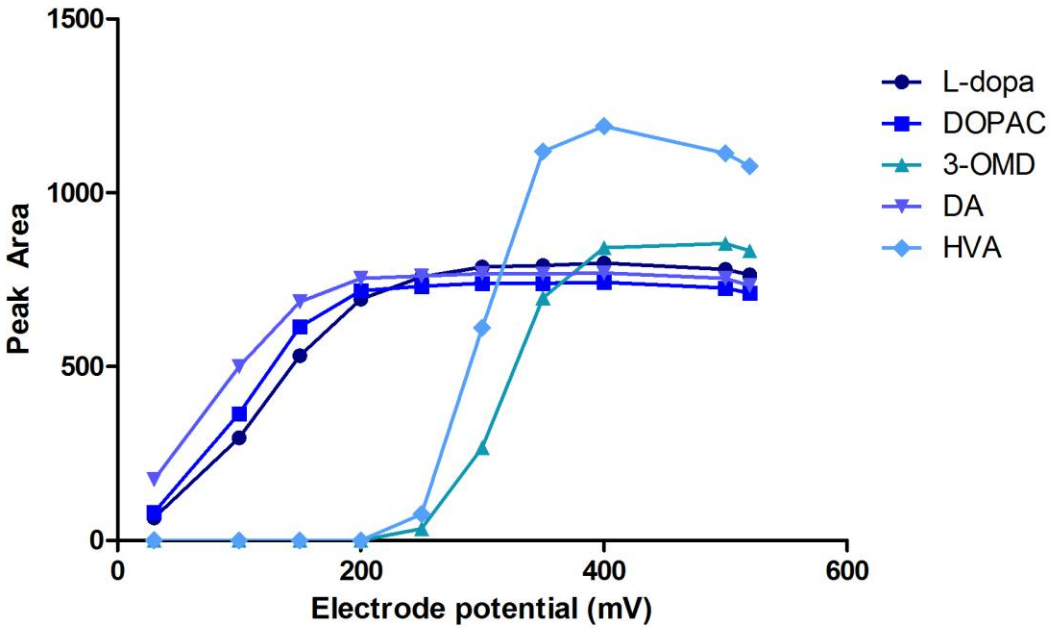


Figure 2.1 Flow diagram of HPLC-ECD system. Used for quantitation of catecholamines, indoleamines and GSH

a.



b.

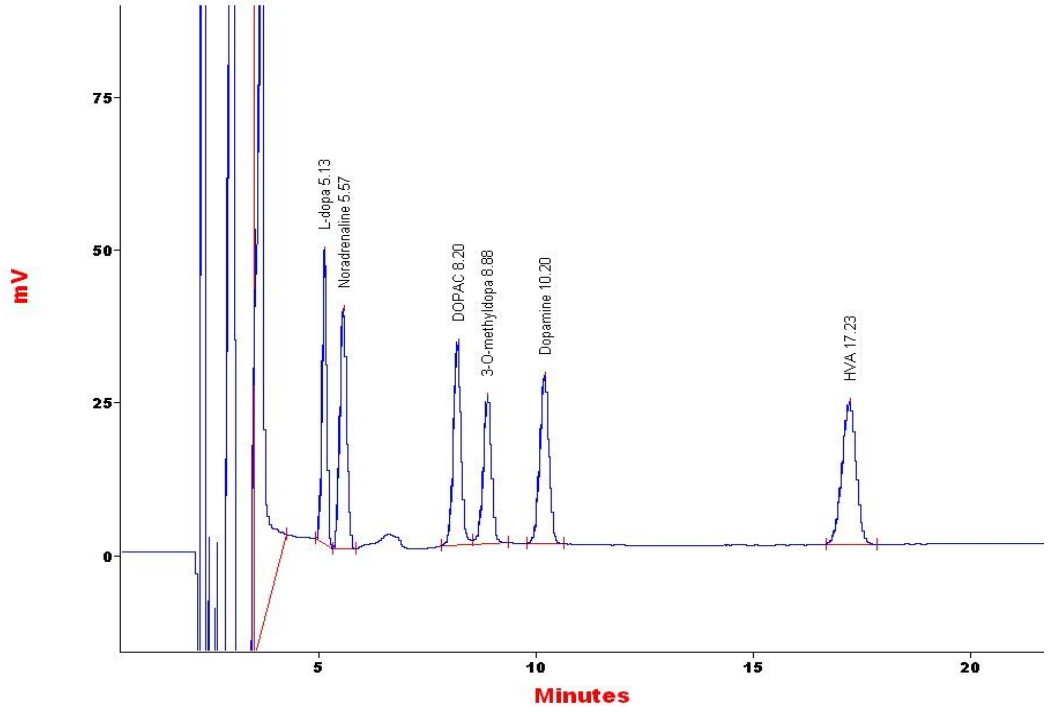
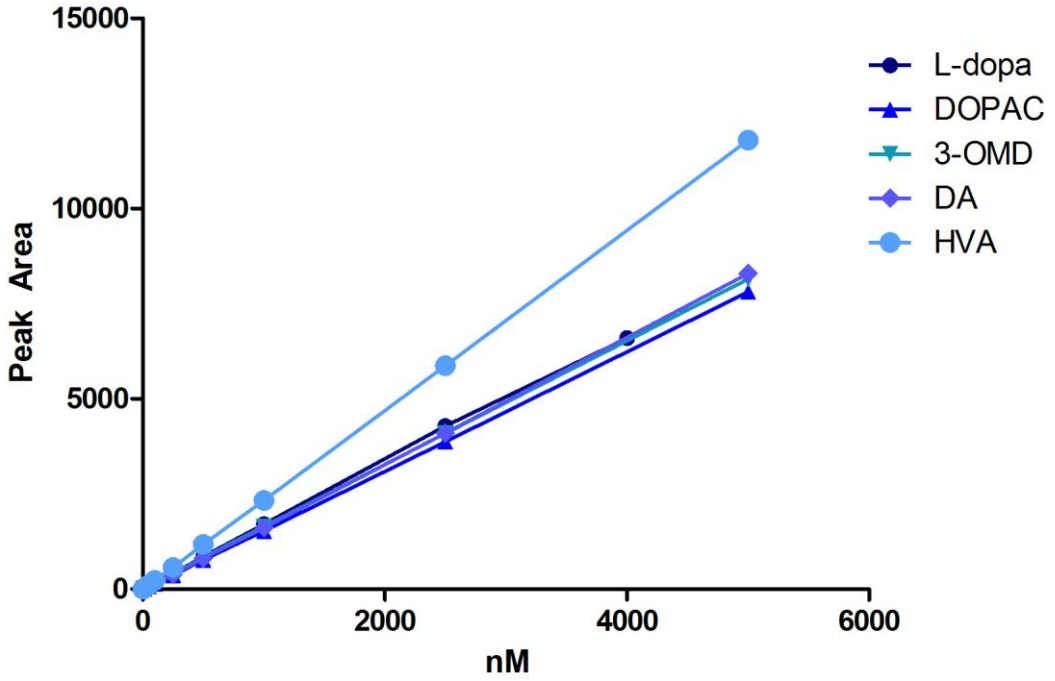


Figure 2.2 Caetcholamine HPLC voltamogram and external standard Chromatogram. a. Voltamogram of 250nM L-dopa, 3-OMD, dopamine, DOPAC and HVA. b. Representative chromatogram of an external catecholamine standard of 250nM L-dopa, 3-OMD, dopamine, DOPAC, HVA and noradrenaline.

a.



b.

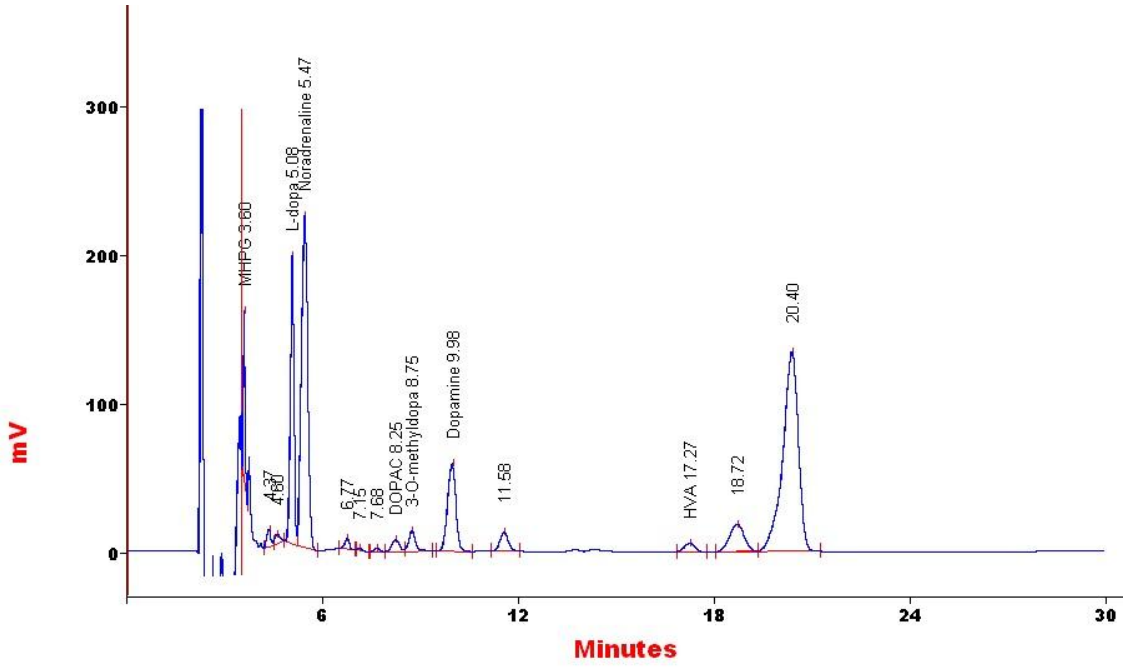


Figure 2.3 Catecholamine HPLC calibration curve and sample chromatogram. a. Caibration curve of L-dopa, 3-OMD, dopamine, DOPAC and HVA. b. Example of a SH-SY5Y sample chromatogram.

+4°C. 50µL of each sample was injected and separated on a C18 reversed phase column (HiQ sil C18W) at +35°C over a run time of ~16 minutes. Serotonin, 5-HTP and 5-HIAA were detected by coulometric electrochemical detection. The screening electrode (E1) was set to +20mV to oxidize analytes with a low oxidation potential. A voltammogram of 500nM serotonin, 5-HTP and 5-HIAA was performed by varying the potential of electrode 2 (E2), the detector electrode, from +150mV to +400mV and determining peak areas (see figure 2.4a). The optimum voltage for the measurement of all three chemicals simultaneously was determined to be +350mV. Serotonin, 5-HTP and 5-HIAA were quantified against an external standard containing 500nM serotonin, 500nM 5-HTP, 500nM 5-HIAA in HPLC grade water with 2 drops 12.2M HCl (see figure 2.4b). A calibration curve for serotonin, 5-HTP and 5-HIAA was also performed. The relationship between indoleamine concentration and current amplitude was found to be linear across the concentration range tested (see figure 2.5a, 5-HIAA r^2 = 1.000, 5-HTP r^2 = 1.000, serotonin r^2 = 1.000). See figure 2.5b for an example of a sample chromatogram. Peaks were identified and quantified using the AZUR Version 4.6 software package (Datalys) as for catecholamines (see section 2.2.2.2).

2.2.4 GSH HPLC

2.2.4.1 Equipment

HPLC equipment: PU-1580 pump (Jasco); AS-2055 Plus autosampler (Jasco); Coulochem II electrochemical detector (ESA) and 5010 analytical cell (ESA) assembled as in figure 2.1. For data recording the electrochemical detector was coupled to a Thermo Finnigan Chromjet integrator (Thermo Fisher Scientific, Rockford, IL, USA). The mobile phase was degassed using a DG-980-50 inline degasser (Jasco).

2.2.4.2 Procedure

GSH was measured using reversed-phase HPLC with coulometric electrochemical detection using the method of Reiderer *et al* (1989). The mobile phase consisted of 15mM orthophosphoric acid in HPLC grade H_2O. The flow rate was 0.5mL/min. Samples were thawed at +20˚C, transferred to vials and loaded into the autosampler and maintained at +20˚C. 50µL of each sample was injected and separated on a C18 reversed phase 250 x 4.6mm i.d. Techsphere ODS column (HPLC Technology, Welwyn Garden City, UK), maintained at +30˚C. The screening electrode (E1) was set to +50mV to oxidise analytes of low oxidation potential. Optimum potential for the

detector electrode (E2) was determined by voltamogram, measuring peak height of 5µM GSH at electrode potentials from +60mV to +650mV (see figure 2.6a). A maximum response was achieved with 600mV, however due to a high background current at this potential an E2 electrode potential of 550mV was chosen for the measurement of GSH. Samples were quantified against an external standard of 5µM GSH in 15mM orthophosphoric acid (see figure 2.6b). A calibration curve was performed to determine the linearity between GSH concentration and current amplitude (see figure 2.7a). Linearity was demonstrated between 0.1µM and 10µM (r^2 = 1.000). An example of a sample chromatogram is presented in figure 2.7b.

2.2.4.3 Data analysis

GSH was identified using a Thermo Finnigan Chromjet integrator (Thermo Fisher Scientific) and quantifed using the following equation:

Conc. (µM) = (sample peak height /external standard peak height) x calibration standard conc. (µM)

For intracellular determination of GSH the conc. (µM) was divided by total protein (mg/mL) and expressed as nmol/mg of protein.

2.2.5 Measurement of PLP by HPLC

Cell samples were processed as described for AADC activity measurement (see section 3.3.2). PLP was measured by Viruna Neergheen and Marcus Oppenheim (Neurometabolic Unit, National Hospital) using a commercial kit (Chromsystems, Munich, Germany). 200µL sample was mixed with 300µL precipitation reagent (Chromsystems) and incubated at +4˚C for 10 minutes. Samples were then centrifuged at 12000xg for 5min at room temperature. 200µL of supernatant was diluted with 200µL neutralisation reagent (Chromsystems), and 80µL derivatisation reagent (Chromsystems) was subsequently added. Samples were then incubated at +60˚C for 20min and subsequently incubated at +4˚C for 10min. Samples were then centrifuged for 2min at 12000xg for 5min at room temperature. Supernatant was transferred to autosampler vials ready for injection onto HPLC.

HPLC equipment was as follows: PU-980 pump (Jasco); AS-950 autosampler (Jasco); FP-920 fluorescence detector (Jasco) assembled as in figure 2.1. The fluorescence detector was coupled to a computer and data recorded using AZUR version 4.6

chromatography data capture and analysis software (Datalys). The mobile phase (Chromsystems) was run at a flow rate of 1.3mL/min. 50µL of each sample was injected onto the column (Chromsystems) which was maintained at +20˚C. PLP was detected by fluorescence detection with an excitation wavelength of 320nm and an emission wavelength of 415nm. Sample PLP was quantified against an external plasma calibration standard (Chromsystems), which varied between batches but was between 47 and 53nM PLP, using AZUR version 4.6 software.

Tissue Culture

2.2.6 SH-SY5Y neuroblastoma cell culture

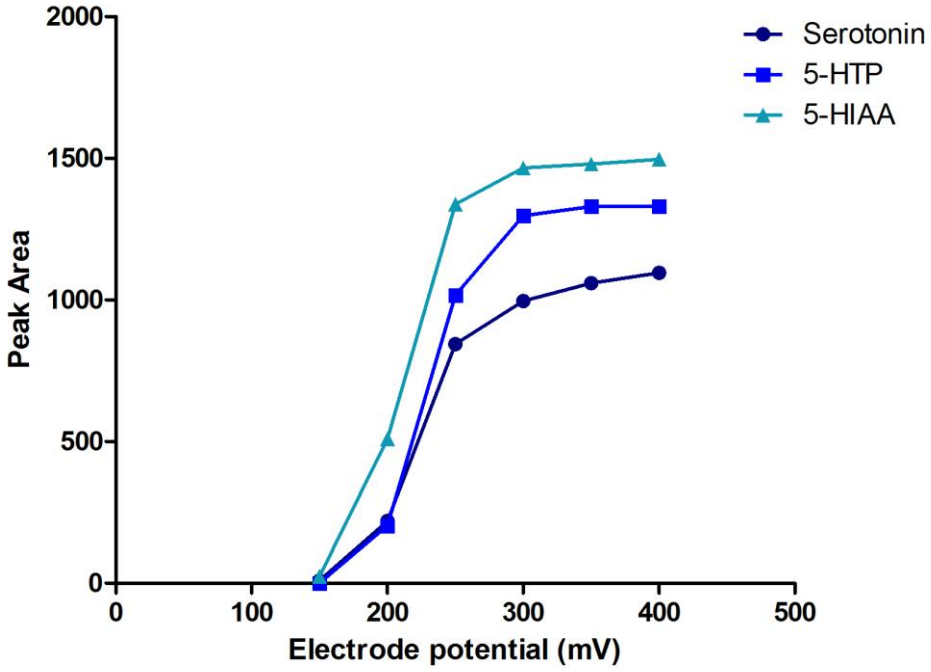
2.2.6.1 Cell line

The SK-N-SH cell line was established from a bone-marrow biopsy of a four year old female patient with a neuroblastoma of the chest (Biedler *et al*, 1973). This line contained two distinct cell groups a neuroblast-like cell and an epithelial-like cell. The neuroblast-like cell group was sub-cloned sequentially three times as SH-SY, then SH-SY5 and finally SH-SY5Y (Biedler *et al*, 1978). The SH-SY5Y cell line has a neuroblast-like morphology (see figure 2.8a), with neurite-like processes, has previously been demonstrated to express tyrosine hydroxylase, dopamine β-hydroxylase, contain AADC mRNA and dopamine (Biedler *et al*, 1978; Ross *et al*, 1980; Oyarce and Fleming, 1991; Gomez-Santos *et al*, 2002; McLaughlin *et al*, 2006). Additionally the kynurenine pathway enzymes can be inducibly expressed in the parental cell line SK-N-SH by the addition of interferon-γ (Guillemin *et al*, 2007).

2.2.6.2 Cell Storage

SH-SY5Y cells were obtained from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and cultured in accordance with supplier's instructions. Cells were seeded at a density of 1 x 10^4 cells/cm^2 in 75cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium/ Ham's F-12 nutrient mixture (1:1; DMEM/F-12; Invitrogen) supplemented with 100mL/L foetal bovine serum (FBS). Cells were grown at +37°C in 5% CO_2, culture medium was replaced the day after seeding and every other day thereafter. Cells were passaged at 80-90% confluence. For passage each flask was washed once with 10mL Hank's balanced salt solution (HBSS) at +37°C. Cells were lifted with 6mL/flask 2.5g/L trypsin, 0.38g/L EDTA in HBSS

a.



b.

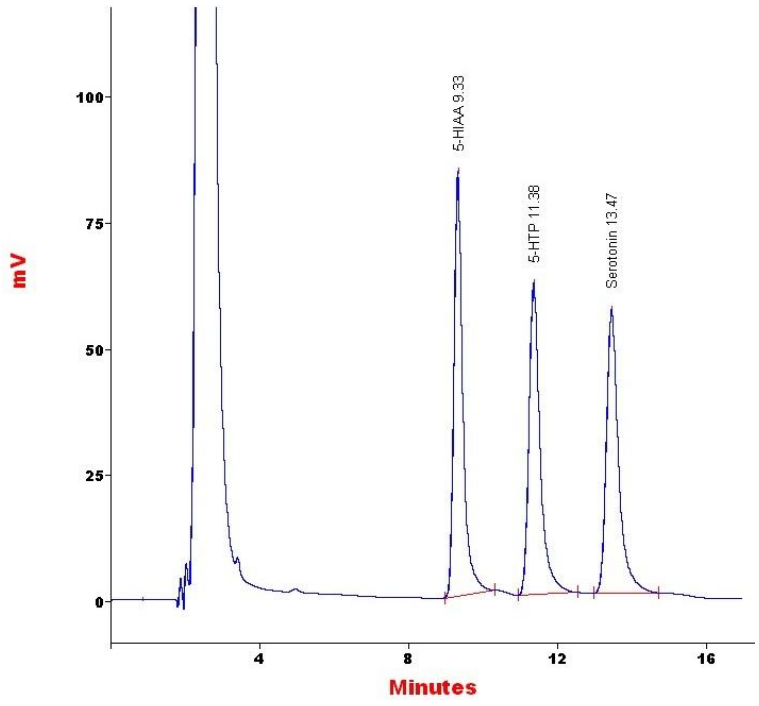
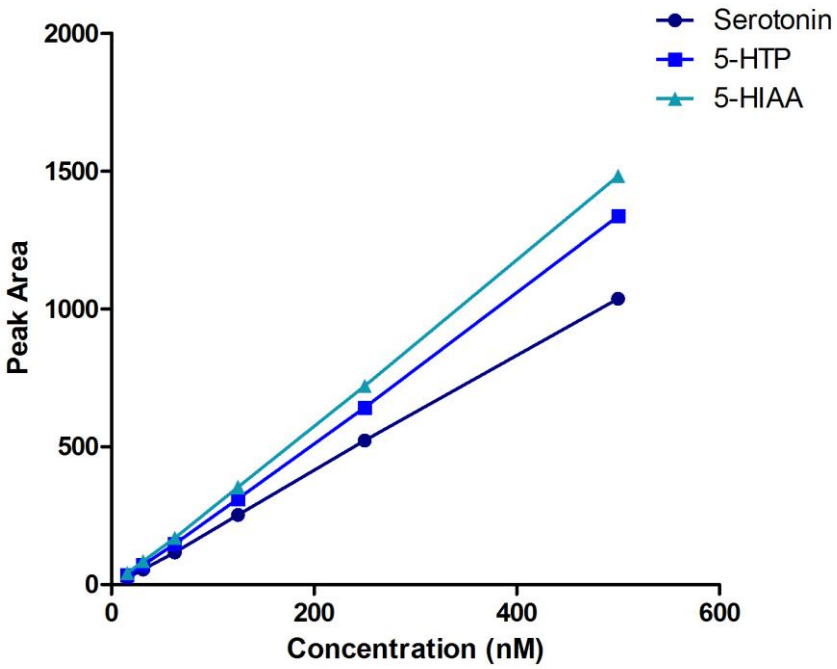


Figure 2.4 Indoleamine HPLC voltamogram and external standard chromatogram. a. Voltamogram of 500nM 5-HTP, serotonin and 5-HIAA. b. Representative chromatogram of an external catecholamine standard of 500nM 5-HTP, serotonin and 5-HIAA.

a.



b.

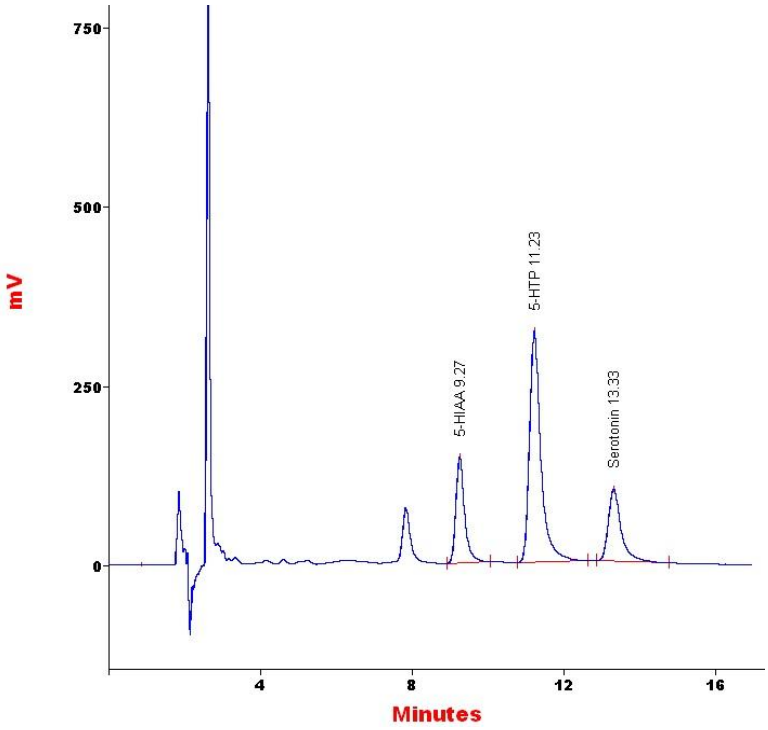
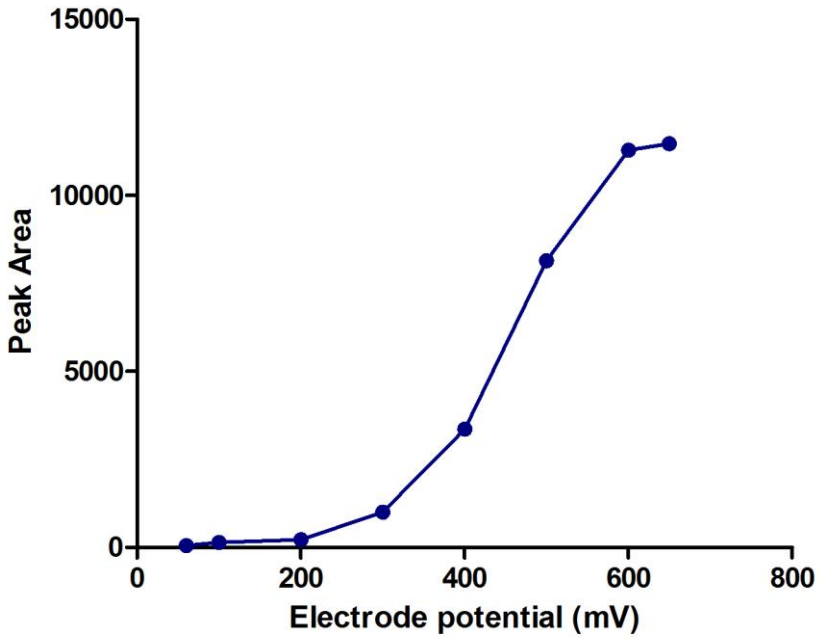


Figure 2.5 Indoleamine HPLC calibration curve and sample chromatogram. a. Caibration curve of 5-HTP, serotonin and 5-HIAA. b. Example of an SH-SY5Y cell sample chromatogram of indoleamine HPLC

a.



b.

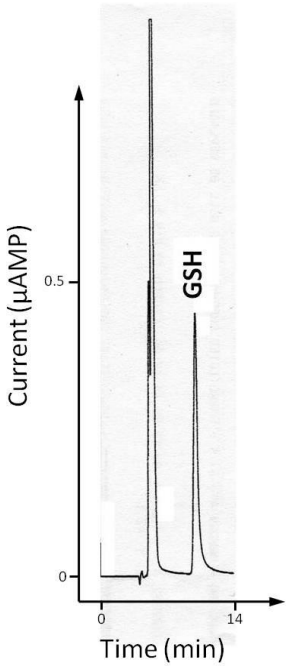
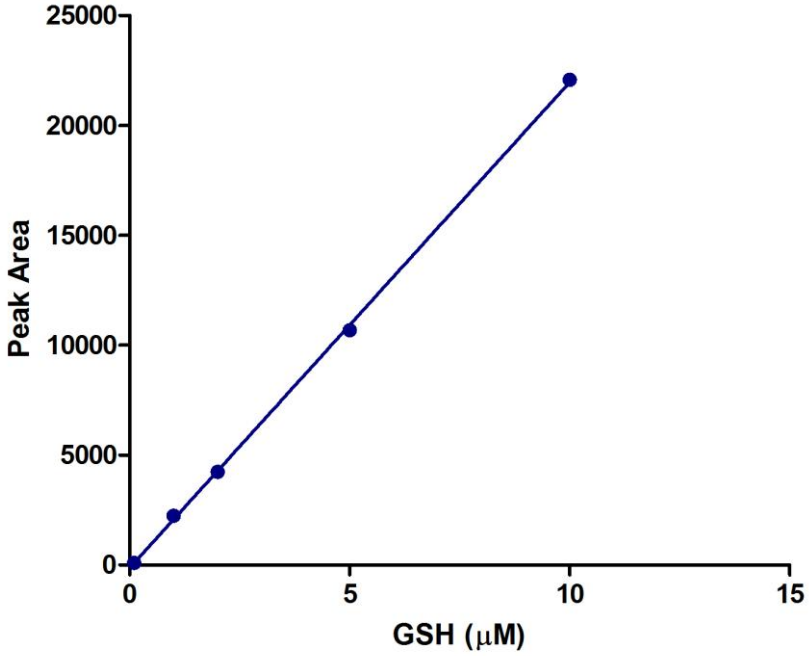


Figure 2.6 Glutathione voltamogram and external standard chromatogram. a. Voltamogram of 5µM GSH. b. Representative chromatogram of an external standard of 5µM GSH.

a.



b.

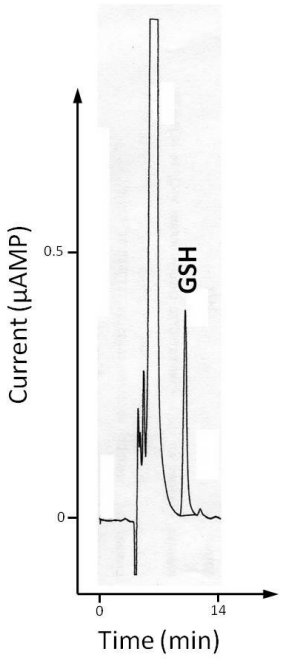


Figure 2.7 Glutathione HPLC calibration curve and sample chromatogram a. Calibration curve of GSH. b. Example of a sample chromatogram of GSH HPLC.

(trypsin-EDTA; Invitrogen) incubated at +37°C for 3min. Cells were collected by centrifugation at 500xg for 5min at +20˚C. Supernatant was removed and cells resuspended in DMEM/F-12 + 100mL/L FBS. 100µL cell suspension was mixed 1:1 with 4g/L trypan blue and cells counted using a Neubauer improved hemocytometer. Cells of passage number 17 to 19 were stored at a density of 1 x 10^6 cells/mL in 300mL/L FBS, 100mL/L dimethyl sulfoxide (DMSO) and 700mL/L DMEM/F-12 as 1mL aliquots. Cells were frozen to -80°C using an isopropanol freezing vessel for 24hr and then transferred to liquid nitrogen for storage.

2.2.6.3 Cell Recovery and Passage

Cells were recovered from liquid nitrogen and seeded at a density of 6.7 x 10^3 cells/cm^2 in 75cm^2 flasks. Cells were grown at +37°C in 5% CO_2 in DMEM/F-12 + 100mL/L FBS. Cell medium was replaced the day after seeding and every other day thereafter. Cells were passaged at 80-90% confluence. For passage cells were washed once with 10mL/flask HBSS. Cells were lifted with 6mL/flask trypsin-EDTA incubated at +37°C for 3min. Cells were collected by centrifugation at 500xg for 5min at +20˚C. Supernatant was removed and cells resuspended in DMEM/F-12 + 100mL/L FBS. 100µL cell suspension was mixed 1:1 with 4g/L trypan blue and cells counted on a Neubauer improved hemocytometer. Cells were seeded at a density of 1 x 10^4 cells/mL in 75cm^2 or 175 cm^2 flasks and grown in DMEM/F-12 + 100mL/L FBS at +37°C in 5% CO_2. Cell medium was replaced the day after seeding and every other day thereafter. Cells were passaged as above at 80-90% confluence approximately every 6 days. To maintain consistency cells of passage number 19 to 24 were used for experiments.

2.2.7 1321N1 astrocytoma cell culture

2.2.7.1 Cell line

The 1321N1 cell line was originally derived from a human brain glioblastoma culture designated 118 MG that was extracted from a 50 year old male (Ponten and Macintyre, 1968). 118 MG was subcloned as 1181N1 and finally as 1321N1 (Macintyre *et al*, 1972; Foster and Perkins, 1977). 1321N1 cells have an astrocyte-like morphology (see figure 2.8b) and express the glial specific marker vimentin as well as the astrocyte specific markers glial fibrillary acidic protein and S-100 (Mead and Pentreath, 1998).

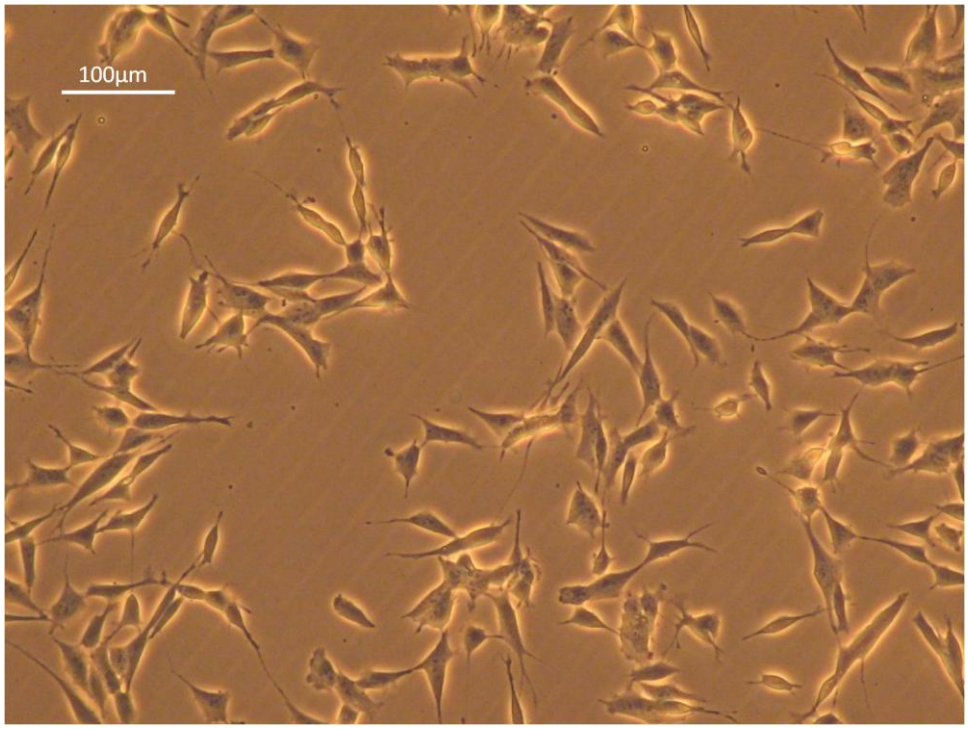
2.2.7.2 Cell storage

1321N1 cells were obtained from the European Collection of Cell Cultures (Health Protection Agency) and cultured in accordance with supplier's instructions. Cells were seeded at a density of 3.4 x 10^4 cells/cm^2 in 75cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100mL/L FBS. Cells were grown at +37˚C in 5% CO_2, the culture medium was replaced the day after seeding and every other day thereafter. Cells were passaged at 70-80% confluence. For passage each flask was washed once with 10mL HBSS at +37°C. Cells were lifted with 6mL/flask trypsin-EDTA incubated at +37°C for 3min. Cells were collected by centrifugation at 500xg for 5min at +20˚C. Supernatant was removed and cells resuspended in DMEM + 100mL/L FBS. 100µL cell suspension was mixed 1:1 with 4g/L trypan blue and cells counted using a Neubauer improved hemocytometer. Cells of passage number 6 to 8 were stored at a density of 2 x 10^6 cells/mL in 300mL/L FBS, 100mL/L DMSO and 700mL/L DMEM as 1mL aliquots. Cells were frozen to -80°C using an isopropanol freezing vessel for 24hr and then transferred to liquid nitrogen for storage.

2.2.7.3 Cell Recovery and Passage

Cells were recovered from liquid nitrogen and seeded at a density of 1.4 x 10^4 cells/cm^2 in 75cm^2 flasks in DMEM + 100mL/L FBS. Cells were grown at +37°C in 5% CO_2. Cell medium was replaced the day after seeding and every other day thereafter. Cells were passaged at 70-80% confluence. For passage cells were washed once with 10mL/flask HBSS. Cells were lifted with 6mL/flask trypsin-EDTA incubated at +37°C for 3min. Cells were collected by centrifugation at 500xg for 5min at +20˚C. Supernatant was removed and cells resuspended in DMEM + 100mL/L FBS. 100µL cell suspension was mixed 1:1 with 4g/L trypan blue and cells counted on a Neubauer improved hemocytometer. Cells were seeded at a density of 1 x 10^4 cells/mL in 75cm^2 or 175 cm^2 flasks and grown in DMEM + 100mL/L FBS at +37°C in 5% CO_2. Cell medium was replaced the day after seeding and every other day thereafter. Cells were passaged as above at 80-90% confluence approximately every 3 days. To maintain consistency cells of passage number 7 to 12 were used for experiments.

a.



b.

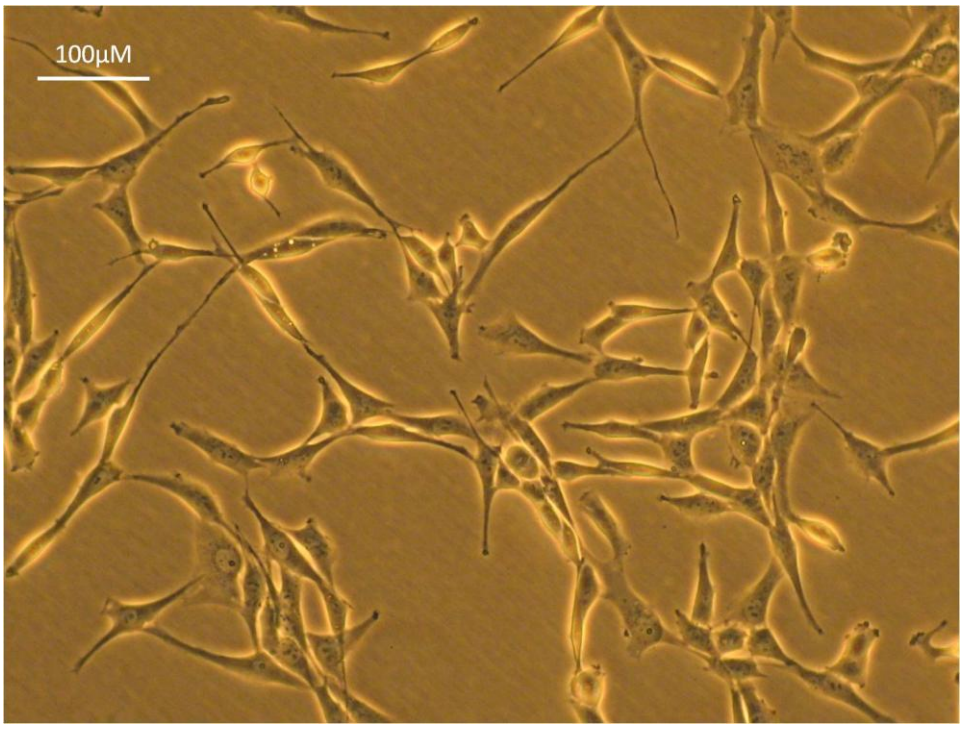


Figure 2.8 Cell images. a. SH-SY5Y neuroblastoma cells b. 1321N1 astrocytoma cells viewed under a light microscope

2.2.8 Cell sample collection

2.2.8.1 SH-SY5Y cell and media harvesting for catecholamine and indoleamine HPLC

Following treatment 500µL cell culture media was sampled and centrifuged at 500xg for 5min at +4°C. Supernatant was stored at -80°C. Immediately prior to analysis samples were thawed at +20˚C, diluted 1:1 in 0.8M perchloric acid and incubated at +4°C for 10min. Samples were centrifuged at 12000xg for 5min at +4°C. Supernatant was analysed by HPLC as described in section 2.2.1 or section 2.2.3. Subsequent dilutions where required were made using HBSS.

Cells were washed once with HBSS, lifted with trypsin-EDTA at +37°C for 3 min. Trypsin was quenched by the addition of FBS. Cells were collected by centrifugation at 500xg for 5min at +4˚C. The supernatant was removed and the cells washed by resuspension in HBSS at +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C. The supernatant was removed and cells were resuspended in isolation buffer: 10mM tris (pH7.4), 1mM EDTA, 320mM sucrose in HPLC grade H_2O. Cell samples were stored at -80°C. Immediately prior to analysis samples were thawed at +37°C and subsequently snap frozen in liquid nitrogen and thawed at +37°C twice to lyse cells. Samples were diluted 1:1 in 0.8M perchloric acid and incubated at +4°C for 10min and subsequently centrifuged at 12000xg for 5min at +4°C. Supernatant was analysed by HPLC as described in section 2.2.1. or section 2.2.3

2.2.8.2 SH-SY5Y and 1321N1 cell harvesting for GSH HPLC

Following treatment cells were washed once with HBSS and lifted with trypsin-EDTA at +37°C for 3 min. Trypsin was quenched by the addition of FBS. Cells were collected by centrifugation at 500xg for 5min at +4˚C. The supernatant was removed and the cells washed by resuspension in HBSS at +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C. The supernatant was removed and cells were resuspended in isolation buffer: 10mM tris (pH7.4), 1mM EDTA, 320mM sucrose in HPLC grade H_2O. Cell samples were stored at -80°C. Immediately prior to analysis samples were thawed at +37°C and subsequently snap frozen in liquid nitrogen and thawed at +37°C twice to lyse cells. Samples were diluted 1:1 in 15mM orthophosphoric acid and incubated at +4°C for 15min and subsequently centrifuged at 14000xg for 5min at +4°C. Supernatant was analysed by HPLC as described in section 2.2.4.

2.2.8.3 Minimal medium harvesting for GSH HPLC

Minimal medium consisted of 44mM sodium bicarbonate, 110mM sodium chloride, 1.8mM calcium chloride, 5.4mM magnesium sulfate, 0.92mM sodium phosphate monobasic and 5mM D-glucose, adjusted with CO_2 to pH7.4. Following incubation with cells minimal medium was removed and transferred to +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C to remove any cells. Supernatant was diluted 1:1 with 15mM orthophosphoric acid and incubated at +4˚C for 15min to precipitate protein. Samples were then centrifuged at 14000xg for 5min at +4˚C. Supernatants were stored at -80˚C until analysis by HPLC as described in section 2.2.4.

2.2.8.4 SH-SY5Y cell harvesting for western blotting

Following treatments SH-SY5Y cells were transferred to ice and washed once with HBSS at +4˚C. Cells were collected by scraping into 10mM tris (pH8.0), 150mM NaCl, 20g/L CHAPS buffer containing protease inhibitor cocktail at manufacturer's recommended concentration (Sigma) at +4˚C and subsequently snap frozen in liquid nitrogen and stored at -80°C. Immediately prior to analysis samples were thawed and agitated for 1hr at +4°C and then centrifuged at 12000xg for 10min at +4°C. Supernatant was analysed as described in section 2.5.

2.2.8.5 SH-SY5Y cell harvesting for mitochondrial respiratory chain complex assays

Following treatment cells were washed once with HBSS and lifted with trypsin-EDTA at +37°C for 3min. Trypsin was quenched by the addition of FBS. Cells were collected by centrifugation at 500xg for 5min at +4˚C. The supernatant was removed and the cells washed by resuspension in HBSS at +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C. The supernatant was removed and cells were resuspended in isolation buffer: 10mM tris (pH7.4), 1mM EDTA, 320mM sucrose in HPLC grade H_2O. Each cell sample was stored at -80°C in five aliquots, one for each complex, one for citrate synthase and one for total protein. Immediately prior to analysis an aliquot was thawed at +37°C and subsequently snap frozen in liquid nitrogen and thawed at +37°C twice to lyse cells. Samples were analysed as described in section 2.7.

2.3 Sulforhodamine B cell proliferation assay

The Sulforhodamine B (SRB) cytotoxicity assay is based on the published methods of Skehan *et al* (1990) and Vichai and Kirtikara (2006). In this assay cells are grown and treated in 96 well plates. Following treatment cells are fixed and dyed with the protein

dye SRB. The dyed cells are then solubilised and absorbance is measured at 490nm. The absorbance is proportional to the quantity of cells following treatment. This is then compared to cells that were plated at the same time as the treated cells but were fixed at the beginning of treatment (time 0). The advantage of this assay is that it allows for quantitation of the change in the amount of cell material over the treatment period, rather than measurement only of the end point following treatment.

2.3.1 SRB assay procedure

Time 0 and treated 96-well plates were seeded concurrently at the same density. Blank wells were performed on each plate where only DMEM/F-12 + 100mL/L FBS was added to the wells. Treatments were added 24hr after seeding and at this time point 100µL 100g/L trichloroacetic acid (TCA) at +4°C was added to all wells of time 0 plates only and time 0 plates were incubated at +4°C for 1hr. Time 0 plates were then washed four times with HPLC grade H_2O and left to air dry. Treated plates were treated and incubated as indicated (see section 4.4.2 and 5.4.2). Following treatment 100µL 100g/L TCA at +4°C was added to all wells of treated plates and incubated at +4°C for 1hr. Treated plates were washed four times with water and left to air dry for 16hr. 100µL 0.57g/L SRB was added to each well including wells of time 0 plates and incubated at +20˚C for 1hr. Plates were washed four times with 10g/L acetic acid and left to air dry for 16hr. 200µL 10mM tris base unbuffered in HPLC grade H_2O was added to each well and agitated for 15min at +20˚C. Plates were read at 490nm using a FLUOstar omega plate reader (BMG Labtech Ltd, Aylesbury, UK). Results are expressed as the change in absorbance at 490nm following treatment compared to that at time 0 (ΔA490), calculated using the following equation:

$$\Delta A490 = (\text{mean treated} - \text{mean blank}) - (\text{mean time 0} - \text{mean time 0 blank})$$

Where all values are mean absorbance units measured at 490nm.

2.3.2 Validation

This assay has not previously been reported to have been used with SH-SY5Y cells and so consequently some validation was undertaken. For validation cells were plated in 96 well plates and allowed to adhere for two hours at +37°C in 5% CO_2 before being fixed, dyed and measured as described above (see section 2.3.1). It is essential for the SRB assay that a supramaximal dye concentration and a sufficient dye incubation time are used to ensure that all cell protein is dyed. Six different amounts of SRB were tested

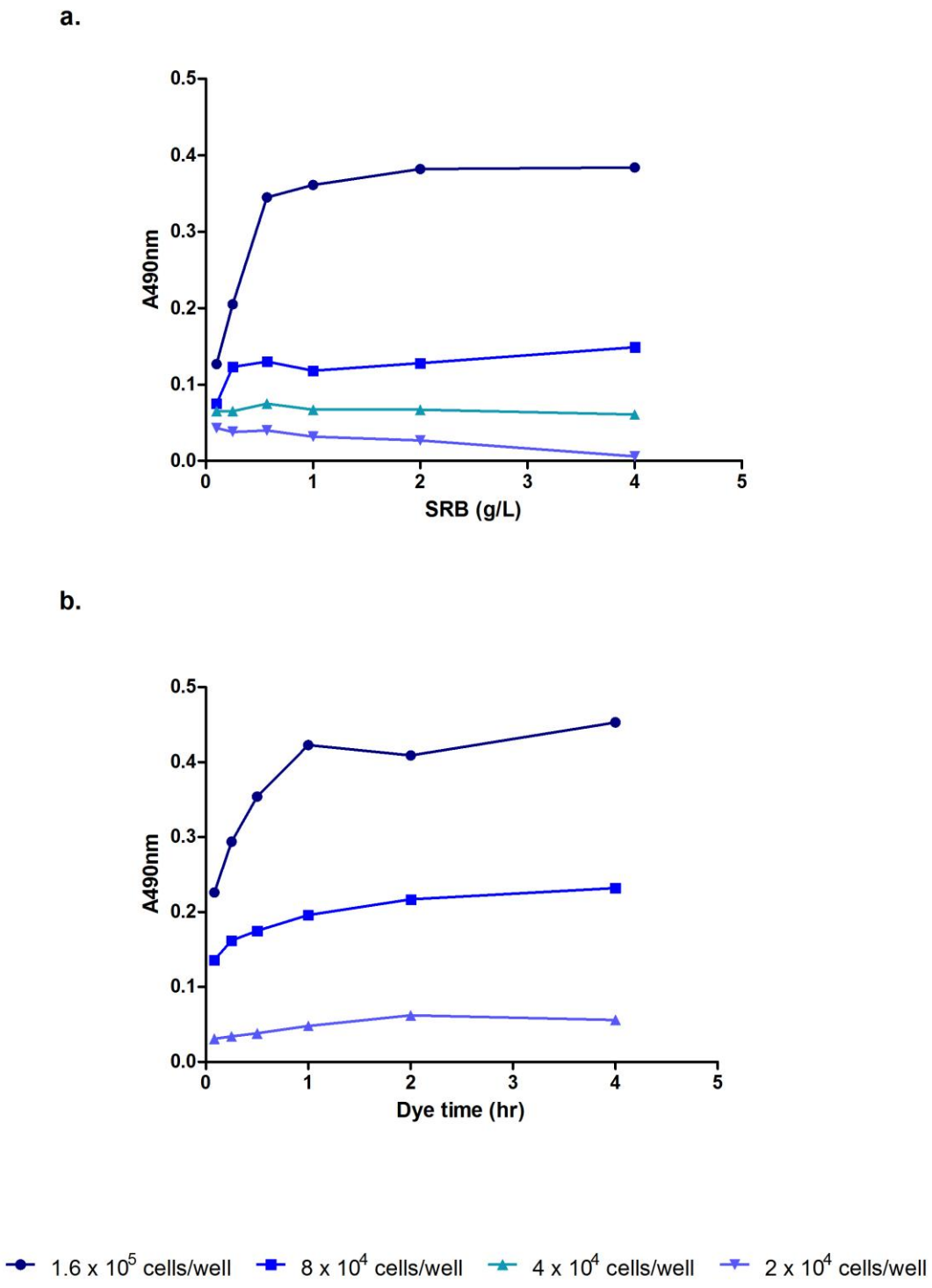


Figure 2.9 SRB assay validation of dye amount and time. a. Effect of different percentages of SRB on absorbance at 490nm with different densities of cells b. Effect of SRB incubation time on absorbance at 490nm with different cell densities.

across a single plate from 0.1g/L to 4.0g/L, at four different cell densities from 2.0 x 10^4 cells/well to 1.6 x 10^5 cells/well, each well was performed in quadruplicate and dyed for 1hr. 0.57g/L SRB was chosen for future experiments as cells were saturated across the range at this dye level (see figure 2.9a). Six different dye incubation times were tested from 0.5hr to 4hr using 0.57g/L SRB, at three different cells densities from 2.0 x 10^4 to 1.6 x 10^5 across a single plate. Wells were performed in quadruplicate. A dye incubation time of 1hr was chosen for future experiments, as cells appeared to be saturated by this time (see figure 2.9b). The linearity of the relationship between cell number and absorbance was also established by testing from 2500 cells/well to 2 x 10^5 cells/well. Seven wells were performed per cell density across one plate. Using 0.57g/L SRB and incubating for 1hr the relationship was linear across the cell density range tested (see figure 2.10a; $r^2 = 0.9881$).

2.4 Lactate dehydrogenase release assay

The release of the cytosolic enzyme lactate dehydrogenase (LDH) into cell culture media can be used as a measure of membrane integrity or cell lysis. This method measures the oxidation of NADH, as the reduction in absorbance at 340nm, during the conversion of pyruvate to lactate by LDH. This method is based on that of Decker and Lohmann-Matthes (1988) where LDH activity in cell culture media as a proportion of total LDH activity in cells and media combined is used to give a quantitative measure of cell lysis.

2.4.1 LDH Release assay procedure

For each plate 10g/L triton X-100 was added to 4 wells at the same time as treatments as a positive control. Following treatment 50µL of media was removed from each well of 96 well plate and transferred to a new plate (plate A). 50µL 40mL/L triton X-100 was added to original plate and plate agitated for 30min at +20˚C to lyse cells. Meanwhile 50µL 80mM Tris (pH7.2), 200mM NaCl was added to each well of plate A. 100µL 80mM Tris (pH7.2), 200mM NaCl, 0.4mM β-NADH, 3.2mM sodium pyruvate was added to each well of plate A and plate read at 340nm over 20min at 1min intervals at +30˚C using FLUOstar omega plate reader (BMG Labtech Ltd). After the 30min incubation with triton X-100, 50µL from each well of original plate was transferred to a new plate (plate B). 50µL 80mM Tris (pH7.2), 200mM NaCl was added to each well of plate B. 100µL 80mM Tris (pH7.2), 200mM NaCl, 0.4mM β-NADH, 3.2mM pyruvate was added to each well of plate B and plate read at 340nm over 20min at 1min intervals

at +30˚C using a FLUOstar omega plate reader (BMG Labtech Ltd). For each assay a linear portion of the reaction was chosen between 6 and 10min in length. The same time frame was used for plate A and plate B within each assay. The change in absorbance at 340nm was calculated for each plate. To take account of the dilution factor of the triton X-100 plate B ΔA340 was multiplied by 1.25. Mean values from blank wells containing only media performed on each plate were then subtracted from each mean value. Results are expressed as LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis (% LDH release).

2.4.2 Validation

The LDH release assay has been used previously with SH-SY5Y cells (Lai and Yu, 1997) however the linearity of LDH activity with cell number was determined to ensure the assay was fit for purpose. Cells were diluted to the appropriate density and seeded in 96 well plates and allowed to adhere for two hours at +37˚C in 5% CO_2. 50µL of cell culture media was removed from each well and 50µL 40mL/L triton X-100 was added to each well. The plate was agitated at +20˚C for 30min to lyse cells. The LDH assay was performed as described above for plate B and LDH activity calculated. The relationship between cell number and LDH activity was found to be linear from 0.05 x 10^5 to 1.2 x 10^5 cells/well (r^2 = 0.991; see figure 2.10b).

2.5 Western Blotting

Samples were diluted to 10µg of protein/30µL + 10µL of 4xNuPAGE sample buffer (Invitrogen) containing 1mM dithiothreitol. All samples were boiled for 5min at +100°C. Samples were separated on a 4-12% Bis-Tris gel in NuPAGE MES SDS running buffer (Invitrogen) at +150V on ice for ~2hr. Protein was transferred to immobilon-P polyvinylidene fluoride (PVDF) membrane 0.45µm (Millipore, Billerica, MA, USA) by electrotransfer, in 25mM Tris (pH8.3), 192mM glycine, 200mL/L methanol transfer buffer at +80V for 70min. All antibodies were diluted in blocking buffer (30g/L non-fat dry milk in phosphate buffered saline with 0.5mL/L tween-20 (PBST)). Following blocking for 1hr in blocking buffer, membranes were probed with primary goat polyclonal to human AADC (Abcam, Cambridge, UK; 1:5000) or β-actin mouse monoclonal (Sigma; 1:10000) for 16hr at +4°C, washed 3 x 20min in PBST, then re-blocked for 20min in blocking buffer. Membranes were then incubated with secondary antibody either horseradish peroxidise (HRP) conjugated polyclonal to goat

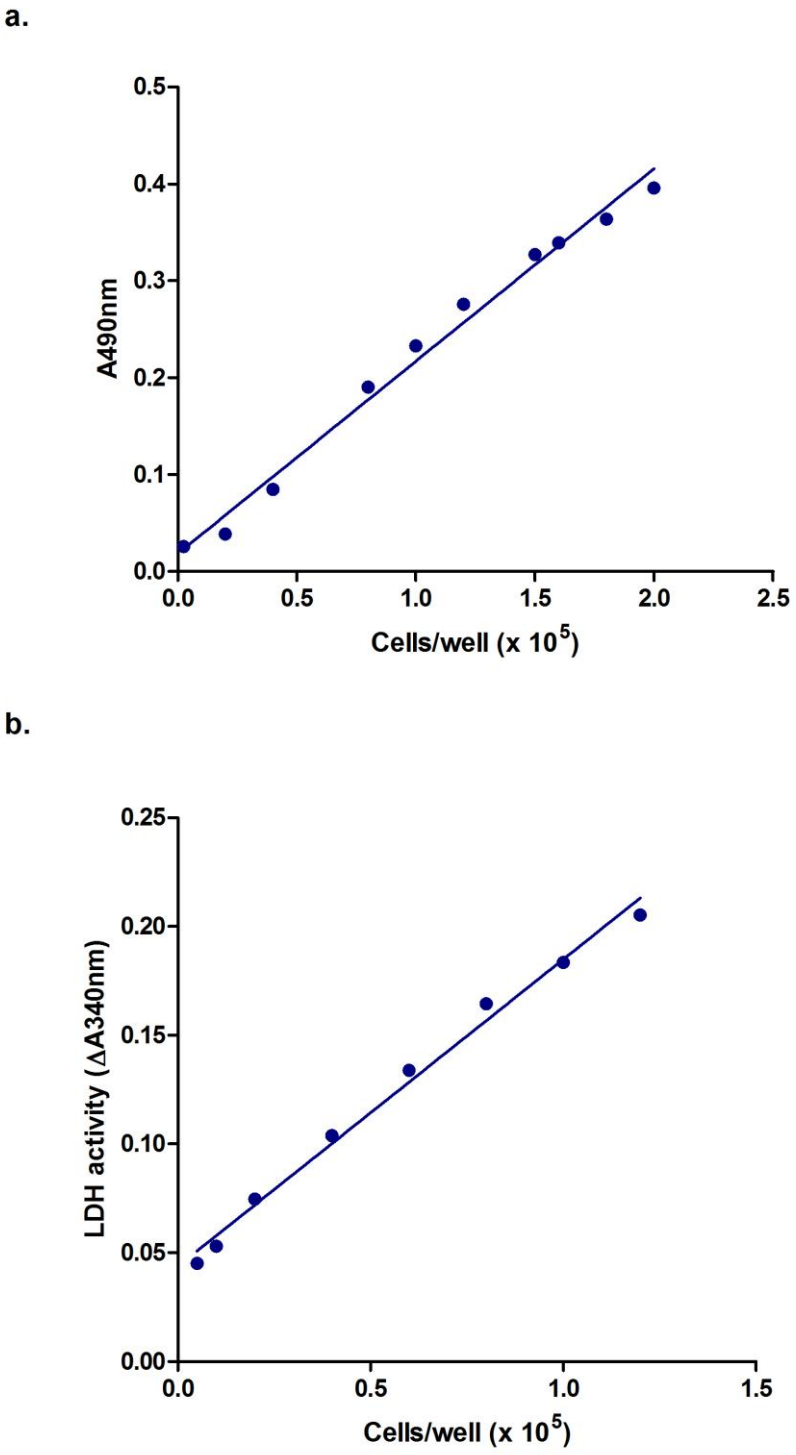


Figure 2.10 Linearity with cell number for SRB and LDH release. a. Relationship between cell density and absorbance at 490nm during the SRB assay, b. Relationship between cell density and LDH activity measured as the change in absorbance at 340nm during the LDH release assay.

IgG (Abcam; 1:2000) for AADC or HRP conjugated anti-mouse IgG (Sigma; 1:2000) for β- actin and subsequently washed 3 x 20min in PBST. The membranes were incubated with enhanced chemiluminescence HRP substrate (Thermo Fisher Scientific) according to manufacturer's instructions and visualised by exposure to x-ray film. Quantification was performed using the Bio-Rad (Hemel Hempstead, UK) Molecular Imager and Quantity One software.

2.6 RNA analysis

2.6.1 Cell preparation for RNA analysis

Cells were washed once with HBSS, lifted with 2.5mL/L trypsin-EDTA at +37°C for 3 min. Cells were collected by centrifugation at 500xg for 5min at +4˚C. The supernatant was removed and the cells washed by resuspension in HBSS at +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C. The supernatant was removed and cell pellets were stored at -80˚C.

2.6.2 Total RNA extraction

Total RNA was extracted using the RNeasy minikit (Qiagen, Crawley, UK) according to manufacturer's instructions. Cell pellets were resuspended in 300µL RLT buffer (Qiagen) containing 10µL/mL β-mercaptoethanol and applied to qiashredder spin columns (Qiagen). Columns were centrifuged at 16000xg for 2min at +20˚C to lyse cells. The eluate was mixed with 300µL 700mL/L ethanol and applied to RNeasy spin columns (Qiagen). Columns were centrifuged at 12000xg for 30sec at +20˚C. Eluate was discarded, 700µL RW1 buffer (Qiagen) was added to the column and centrifuged at 12000xg for 5min at +20˚C. Eluate was discarded, 500µL RPE buffer (Qiagen) was added to the column and centrifuged at 12000xg for 5min at +20˚C. This step was repeated once. Eluate was discarded and columns were centrifuged at 16000xg for 2min at +20˚C. 35µL RNase free water was added to the column, incubated for 2min at +20˚C and centrifuged at 16000xg for 2min at +20˚C. Total RNA concentration in the eluate was measured by absorbance at 260nm using a nanodrop spectrophotometer (Thermo-Fisher Scientific).

2.6.3 Reverse Transcription

RNA samples were diluted to a concentration of 0.2µg/µL in RNase free water. 1µL dNTPs (Invitrogen) and 1µL Oligod(T)18 (New England Biolabs, Herts, UK) was

added to 10µL of each sample. Samples were heated for 5min at +95˚C and subsequently transferred to ice. 4µL 5x RT-buffer (Invitrogen), 2µL 0.1M dithiothritol and 1µL RNasin (Promega, Madison, WI, USA) added to each sample and heated for 2min at +42˚C. 1µL superscript II (Invitrogen) added to each sample to start reverse transcription and samples were incubated at +42˚C for 70min followed by +70˚C for 15min. Samples were stored at -20˚C until analysis.

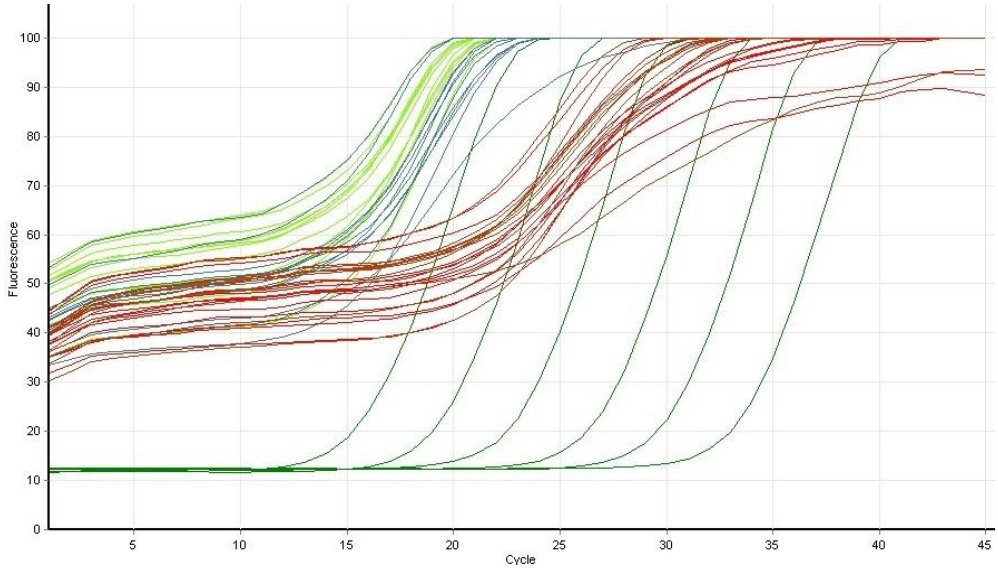
2.6.4 Agarose gel electrophoresis

Reverse transcribed samples were separated on a 10mL/L agarose gel containing 0.2mL/L ethidium bromide in 40mM Tris-acetate, 1mM EDTA (Invitrogen) at 90V for approximately 30min. Samples were run against a 1kb DNA ladder (Promega) and visulised by exposure to UV.

2.6.5 Quantitative polymerase chain reaction (PCR)

Glyceraldehyde 3'-phosphate dehydrogenase (E.C. 1.2.1.9; GAPDH) mRNA was used as a control gene as its expression is not known to be influenced by pyridoxal 5'-phosphate (see section 6.5.2) and is commonly used as a reference gene including in neuroblastoma cells (Dotsch *et al*, 2000; Janssens *et al*, 2004). AADC or GAPDH mRNA expression was quantified against a standard curve ranging from 1 x 10^3 to 1 x 10^8 molecules of GAPDH mRNA in 10mM tris-HCl (pH 8.00), 15mM KCl, 0.6mM $MgCl_2$ in HPLC grade H_2O. The GAPDH mRNA standard was a kind gift from Dr Julia Fitzgerald (Department of Molecular Neuroscience, UCL Institute of Neurology, London). Quantitative PCR was carried out using the Rotor-Gene 6000 system (Qiagen). 5µL iQSybr green supermix (Bio-Rad), 0.3µL each of 10µM forward and reverse primer and 3.4µL HPLC grade H_2O was added to each standard or reverse transcribed sample. The primers used were as follows AADC, 5'-TTC CTT TCT TTA TGG TTG CC-3', 3'-TGT TGG AAC CCT TTA GCC GA-5', GAPDH, 5'-CCA TCA CCA TCT TCC AGG AGC GA-3', 5'-GGA TGA CCT TGC CCA CAG CCT TG-3'. The PCR conditions were denaturation at +95°C for 3min, followed by 45 cycles of denaturation at +94°C for 20sec, annealing at +55°C for 30sec and elongation +72°C for 30sec (see figure 2.11). Data was analysed using rotor-gene software (Qiagen) and AADC mRNA expression is given as arbitrary units/molecule of GAPDH DNA.

a.



b.

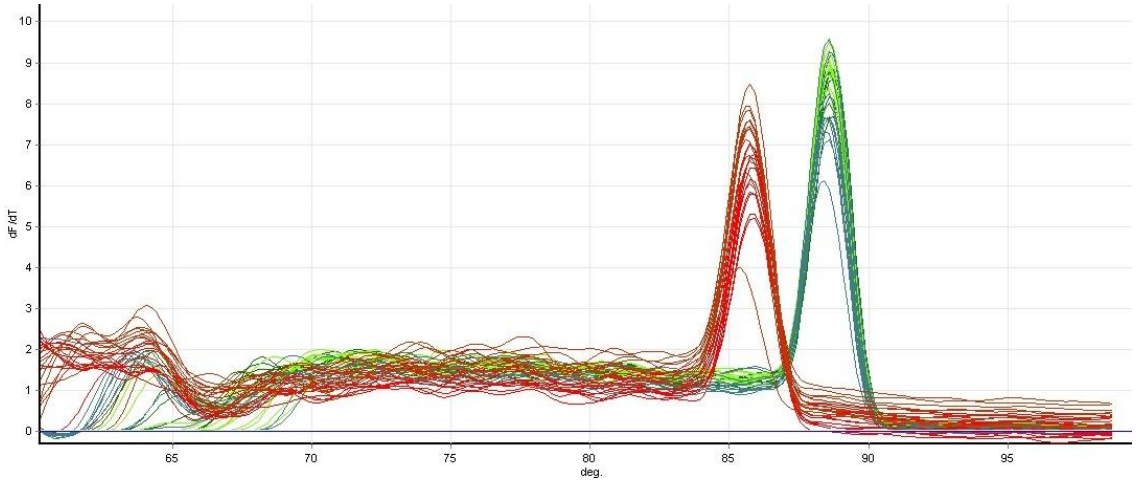


Figure 2.11 Quantitative PCR amplification plot and melt curve. a. A representative amplification plot of fluorescence (ex.470/em.510nm) against PCR cycle number from a quantitative PCR run, red (AADC) and green (GAPDH). Demonstrating accumulation of product across PCR cycles measured by change in dye fluorescence intensity. b. A representative melt curve plotting [change in fluorescence (ex.470/em.510nm)/change in temperature in ˚C (dF/dT)] by temperature in ˚C (deg.). This is a measure of change in dye fluorescence when double stranded DNA dissociates to single stranded DNA, PCR products should be distinguishable as each product dissociates at a specific temperature. Therefore this melt curve demonstrates that only two PCR products were produced, red (AADC) and green (GAPDH), indicating that there was no contamination during the PCR reaction.

2.7 Mitochondrial Respiratory Chain Complex Assays

2.7.1 Complex I

This assay is based on that described by Ragan *et al* (1987) and measures the oxidation of NADH, as the decrease in absorbance at 340nm, during the reduction of ubiquinone to ubiquinol by complex I. The specific complex I inhibitor rotenone is used to determine the proportion of NADH oxidation which is independent of complex I. 20µL of each sample was added to a cuvette containing at final concentration 2.5mg/mL bovine serum albumin (BSA), 0.15mM β-NADH, 1mM potassium cyanide (KCN) in 20mM potasium phosphate (pH 7.2), 8mM magnesium chloride in HPLC grade H_2O.

The reaction was started by the addition of 10µL 5mM ubiquinone (final conc. 50µM), the final volume in each cuvette was 1mL. The reaction was measured at 340nm at 30sec intervals for 5min at +30˚C using a Uvikon 941 spectrophotometer (Northstar Scientific, Potton, UK). 20µL 1mM rotenone (final conc. 20µM) was added to each sample cuvette and measurement was continued for 5min. Each sample was run against a reference cuvette containing all components of the sample cuvette except ubiquione. To produce a specific value for complex I the change in absorbance at 340nm following rotenone addition was subtracted from that before rotenone inhibition. Absorbance was converted to molar concentration using the NADH extinction coefficient 6.81 x 10^3 M^{-1} cm^{-1} (path length 1cm, total volume 1mL), taking account of ubiquinone, using a re-arrangement of Beer-Lambert law:

$$\frac{\Delta A}{\varepsilon} = c$$

Where ΔA is the specific change in absorbance; ε = extinction coefficient; and c = mole/min/mL. Results are expressed as nmol/min/mg of protein or as a ratio to citrate synthase activity.

2.7.2 Complex II/III

Complex II/III was measured by the method of King (1967). Complex II reduces ubiquinone to ubiquinol during the oxidation of succinate to fumarate. Ubiquinol subsequently acts as an electron donor during the reduction of cytochrome c catalysed by complex III. This assay measures the succinate dependent reduction of cytochrome c by measuring the increase in absorbance at 550nm. The complex III inhibitor antimycin A is used to determine the proportion of cytochrome c oxidation which is independent

of complexII/III activity. 20µL of each sample was added to a cuvette containing at final concentration 0.3mM EDTA, 1mM KCN, 0.1mM cytochrome c in 100mM potassium phosphate (pH 7.4) in HPLC grade H_2O. The reaction was started by the addition of 40µL 0.5M succinate (final conc. 20mM), the final volume in each cuvette was 1mL. The reaction was measured at 550nm at 30sec intervals for 5min at +30˚C using a Uvikon 941 spectrophotometer (Northstar Scientific). 10µL 1mM antimycin A (final conc. 10µM) was added to each sample cuvette and measurement was continued for 5min. Each sample was run against a reference cuvette containing all components of the sample cuvette except succinate. To produce a specific value for complex II/III the change in absorbance at 550nm following antimycin A addition was subtracted from that before antimycin A inhibition. Absorbance was converted to molar concentration using Beer-Lambert law (see section 2.7.1) and the extinction coefficient of cytochrome c, 19.2 x 10^3 M^{-1} cm^{-1} (path length 1 cm, total volume 1mL). Results are expressed as nmol/min/mg of protein or as a ratio to citrate synthase activity.

2.7.3 Complex IV

Complex IV was measured by the method of Wharton and Tzagoloff (1967). This assay measures the reduction of cytochrome c catalysed by complex IV. 0.8M oxidised cytochrome c is first reduced by the addition of a few crystals of ascorbic acid. A PD_{10} desalting column equilibrated with 10mM potassium phosphate (pH7.0) in HPLC grade H_2O was used to remove ascorbate from the reduced cytochrome c. 50µL reduced cytochrome c was added to 950µL H_2O in a sample and a reference cuvette and the sample cuvette was blanked against the reference at 550nm using a Uvikon 941 spectrophotometer (Northstar Scientific). Cytochrome c in the reference cuvette was then oxidsed by the addition of 10µL 100mM ferricyanide (final conc. 1mM). The absorbance was recorded after 1min and used to determine the concentration of reduced cytochrome c using beer-lambert law and the extinction coefficient for cytochrome c, 19.2 x 10^3 M^{-1} cm^{-1} (path length 1cm, total volume 1mL). Cytochrome c to a final concentration of 50µM was then added to sample and reference cuvettes containing at final concentration 10mM potassium phosphate (pH7.0) in HPLC grade H_2O. The sample cuvette was blanked against the reference at 550nm using a Uvikon 941 spectrophotometer (Northstar Scientific) and 10µL 100mM ferricyanide (final conc. 1mM) was added to the reference cuvette to oxidise cytochrome c. The reaction was started by the addition of 20µL sample to the sample cuvette and the change in

absorbance at 550nm was recorded over 3min at +30˚C. The reaction of complex IV with cytochrome c follows first-order kinetics as it is dependent on the concentration of cytochrome c. Activity is therefore expressed as a first order rate constant (k). k is calculated by plotting the natural log of absorbance against time and determining the slope. Results are expressed as k/min/mg of protein or k/mol when divided by citrate synthase activity.

2.7.4 Citrate synthase

Citrate synthase catalyses one step of the the citric acid cycle within the mitochondrial matrix and is commonly used as a measure of mitochondrial enrichment (Almeida and Medina, 1998; Hargreaves *et al*, 1999). This enzyme catalyses the condensation of oxaloacetate and acetyl-coenzyme A to form citric acid and coenzyme A. The assay of Shepherd and Garland (1969) was used to measure citrate synthase activity. This assay measures the production of coenzyme A through a reaction of free coenzyme A with 5, 5'-dithio-bis (2-nitrobenzoic acid) (DNTB). 20µL of each sample was added to a cuvette containing at final concentration 0.1mM acetyl-coenzyme A, 0.2mM DNTB in 100mM tris (pH 8.0), 1g/L triton X-100 in HPLC grade H_2O. The reaction was started by the addition of 10µL 20mM oxaloacetate (final conc. 0.2mM) and the reaction measured at 412nm for 5min at 30sec intervals at +30˚C using a Uvikon 941 spectrophotometer (Northstar Scientific). Absorbance was converted to molar concentration using Beer-Lambert law (see section 2.7.1) and the extinction coefficient of DNTB, 13.6 x 10^3 M^{-1} cm^{-1} (path length 1 cm, total volume 1mL). Results are expressed as nmol/min/mg of protein.

2.8 Total protein determination

Total protein was determined by the Bio-Rad DC-protein assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). This assay is a modified method based on that of Lowry *et al* (1951). The assay was performed as per manufacturer's instructions. Briefly all samples were diluted in HPLC grade H_2O. A 6-point standard line was prepared with BSA in distilled water from 0 to 1mg/mL. 5µL of each standard and sample were added to 3 wells of a 96 well plate. 25µL reagent A (alkaline copper tartrate) and 200µL reagent B (Folin-Ciocalteau phenol) was added to each sample. Samples were incubated for 5min at +20˚C, in the dark. After incubation absorbance of samples was measured at 750nm using a FLUOstar omega plate reader (BMG Labtech Ltd). Total protein was

determined from linear regression of sample absorbance units against the BSA standard line.

2.9 Statistical analysis

With the exception of individual patient data all results are expressed as mean ± standard error of the mean (SEM). Individual comparison of means was made using Student's t-test and was carried out using Graphpad Prism software (Graphpad Software Inc. San Diago, CA, USA). Multiple comparisons were made using ANOVA. Independent variables found to be significant by ANOVA were further analysed by least significant difference (LSD) multiple range test using SPSS (SPSS Inc. Chicago, Il, USA). In all cases $p < 0.05$ was considered significant. Prior to statistical analysis % LDH release and complex activites when expressed as a ratio to citrate synthase were transformed by the following calculation:

$$\text{Arcsin} \sqrt{(\text{ratio})}$$

Where percentage values were first divided by 100 to give a ratio. This transformation yielded data with a normal distribution to enable statistical analysis to be performed (Gegg *et al*, 2003).

Chapter 3

Aromatic L-amino acid decarboxylase activity assay method

3.1 Background

AADC activity was originally determined through measurement of CO_2 evolution from tissue extracts upon addition of L-dopa (Holtz *et al*, 1938; Blaschko, 1942). However non-enzymatic reactions involving L-dopa that do not produce dopamine were also found to produce CO_2 and so this method was considered to be inaccurate (Mackowiak *et al*, 1972). Alternative methods measured the product of decarboxylation of various L-aromatic amino acids spectrophotometrically or fluorometrically, or through radioisotopic measurement after the addition of [^{14}C]-DL-dopa (Lovenberg *et al*, 1962; Mackowiak *et al*, 1972; Sherald *et al*, 1973; Charteris and John, 1975). Nagatsu *et al* (1979) used HPLC-ECD to measure L-dopa and dopamine to improve the sensitivity of AADC activity measurement for use with brain homogenates. A similar method measuring dopamine by HPLC-ECD was later developed for determination of AADC activity in human plasma (Boomsma *et al*, 1986). These two HPLC-ECD methods were adapted for use as a diagnostic tool for investigation of AADC deficiency measuring L-dopa or 5-HTP decarboxylation in liver samples or L-dopa decarboxylation in plasma (Hyland and Clayton, 1992).

3.2 Principle

This assay measures the conversion of L-dopa to dopamine or 5-HTP to serotonin. The sample is initially incubated with excess PLP to ensure available AADC is in the PLP-bound form. The reaction is subsequently initiated by the addition of substrate and terminated after a set incubation time by protein precipitation. HPLC-ECD is then used to quantify dopamine or serotonin.

3.3 Method

3.3.1 Patient Plasma Samples

Patient samples were lithium heparin plasma. All patient samples were tested as part of diagnostic investigations requested by the patient's clinician and after informed consent. Anonymised control samples were from patients found not to have conditions relating to vitamin B_6 or monoamine metabolism used in accordance with accredited Neurometabolic Unit (National Hospital for Neurology and Neurosurgery) procedures.

3.3.2 Cell Homogenate Preparation

Cells were washed once with HBSS, lifted with 2.5mL/L trypsin-EDTA at +37°C for 3 min. Cells were collected by centrifugation at 500xg for 5min at +4˚C. The supernatant was removed and the cells washed by resuspension in HBSS at +4˚C. Samples were centrifuged at 500xg for 5min at +4˚C. The supernatant was removed and cells were resuspended in protein isolation buffer: 10mM tris (pH 7.4), 1mM EDTA, 320mM sucrose buffer containing protease inhibitor cocktail (Sigma) at manufacturers recommended concentration. Samples were stored at -80°C until analysis. Immediately prior to analysis samples were thawed at +37°C and subsequently snap frozen in liquid nitrogen and thawed at +37°C twice to lyse cells.

3.3.3 L-dopa decarboxylation

3.3.3.1 Sample incubation

L-dopa decarboxylation was measured using the method of Hyland and Clayton (1992) with minor modifications. 50µL of plasma or cell lysate was incubated with 70µM PLP in assay buffer (500mM sodium phosphate pH 7.0, 0.167mM EDTA, 39mM dithioerythrietol) for 120min at +37°C. 25µL 20mM L-dopa (final conc. in reaction 2mM), except where stated, in 6mM HCl (final conc. in reaction 0.6mM) was added to the reaction mixture and after mixing was incubated for a further 90min (plasma samples) or 5min (cell lysate) at +37°C. The reaction was stopped with 250µL 0.8M perchloric acid (final volume 500µL). Samples were incubated at room temperature for 10min before centrifugation at 12000xg for 5min at +4°C. A substrate blank was also performed, where each sample was treated in exactly the same manner except that no L-dopa was added during the incubations. Within each assay a sample blank was also incubated in exactly the same manner except that the sample was omitted from the reaction mixture. All samples and blanks were stored at -80°C until analysis of dopamine by HPLC as described below (see section 3.4).

3.3.4 5-HTP decarboxylation

3.3.4.1 Sample incubation

5-HTP decarboxylation was only measured in cell lysates not in plasma samples. This method is also based on that of Hyland and Clayton (1992). Incubations were performed in exactly the same manner as for L-dopa decarboxylation except the assay

buffer consisted of 167mM sodium phosphate pH 8.0, 0.167mM EDTA, 39mM dithioerythrietol and in place of L-dopa 25µL 5mM 5-HTP (final conc. in reaction 0.5mM), except where stated, was added at the same time point. Samples and blanks were stored at -80°C before analysis of serotonin by HPLC as described below.

3.4 HPLC

3.4.1 Equipment

The equipment used was as described for catecholamine measurement see section 2.2.2

3.4.2 Procedure

The chromatography procedure was based on the method of Hyland and Clayton (1992). The mobile phase consisted of 50mM sodium phosphate (pH 3.60), 5mM octanesulfonic acid, 67µM EDTA, 43mM orthophosphoric acid, 230mL/L methanol in HPLC grade water. Samples were thawed at room temperature, 200µL of each sample was transferred to a vial, loaded into the autosampler and maintained at +4°C. The flow rate was 1.2mL/min and column temperature was maintained at +25°C or +35°C for dopamine or serotonin measurement respectively. 50µL of each sample was injected and separated on a 250 x 4.6mm i.d. HiQSil C18W column (KYA Tech. Corp. Tokyo, Japan). Dopamine and serotonin were detected by coulometric electrochemical detection. The screening electrode (E1) was set to +20 mV to oxidize analytes with a low oxidation potential. The optimum voltage of the detector electrode (E2) was determined by voltamogram by measuring the peak area of 1000 nM dopamine or 1000nM serotonin at varying E2 potentials from +50mV to +400mV (see figure 3.1a). A potential of +350mV was chosen for the detection of dopamine or serotonin. Samples were quantified using an external standard of 1000nM dopamine or serotonin in 60mM HCl (see figure 3.2). Calibration curves were performed to ensure linearity between current amplitude and concentration (see figure 3.1b). Linearity was demonstrated across all concentrations tested, from 5nM to 4000nM of dopamine (r^2 = 1.000) and 5nM to 5000nM of serotonin (r^2 = 1.000). Examples of sample chromatograms are presented in figure 3.3.

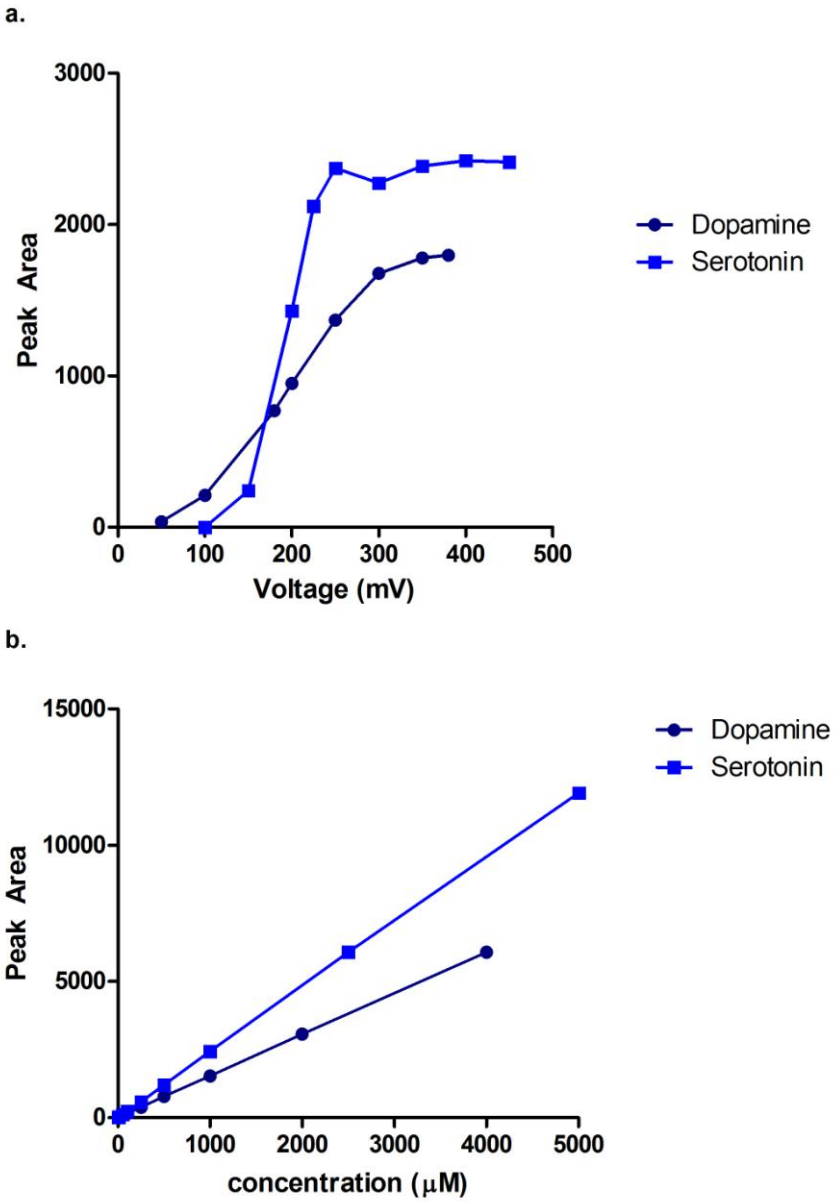
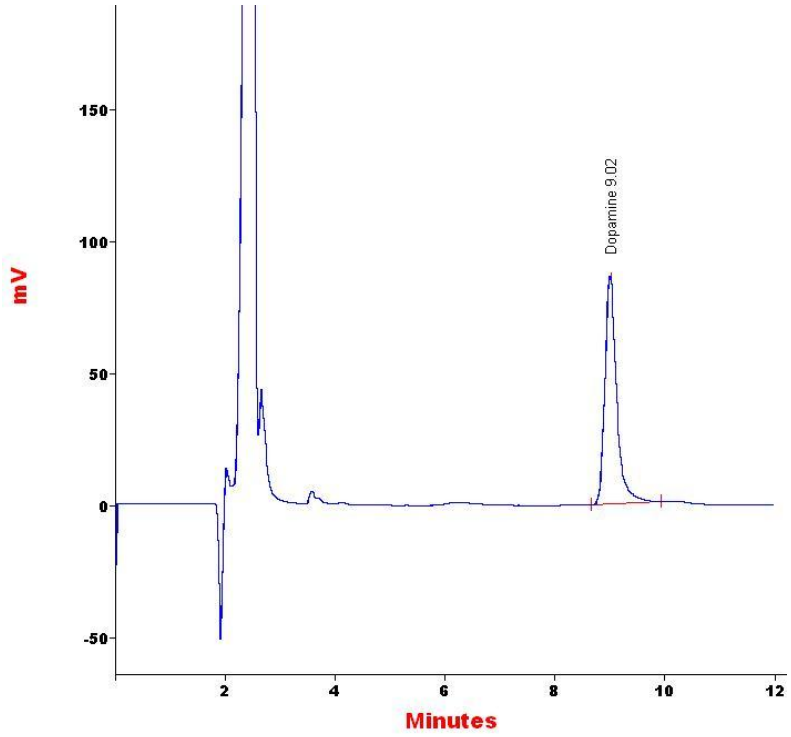


Figure 3.1 Dopamine and serotonin voltamogram and calibration curve for AADC activity assay. a. Voltamogram of 1000nM dopamine and 1000nM serotonin. b. Calibration curve of dopamine and serotonin.

a.



b.

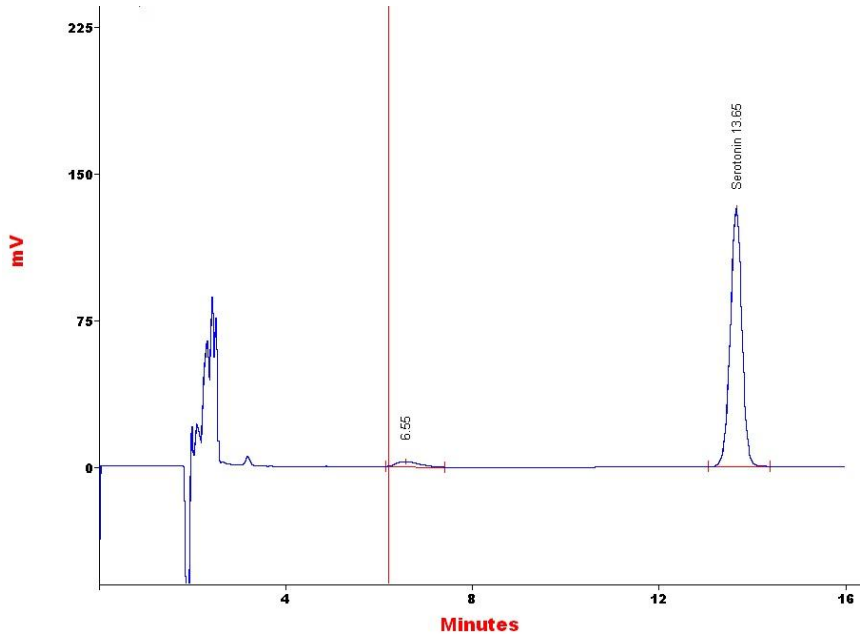
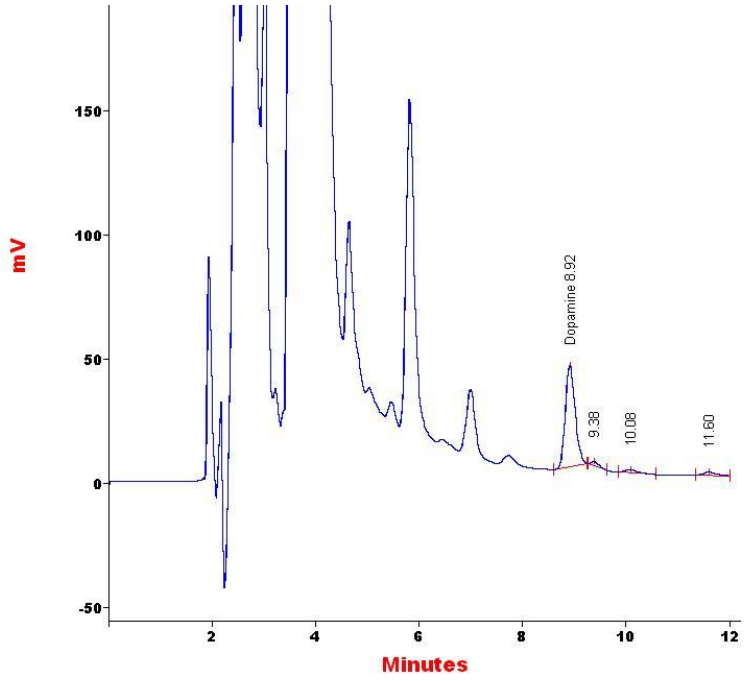


Figure 3.2 External dopamine and serotonin standards for AADC activity assay. Representative chromatogram of an external standard of a. 1000nM dopamine and b. 1000nM serotonin.

a.



b.

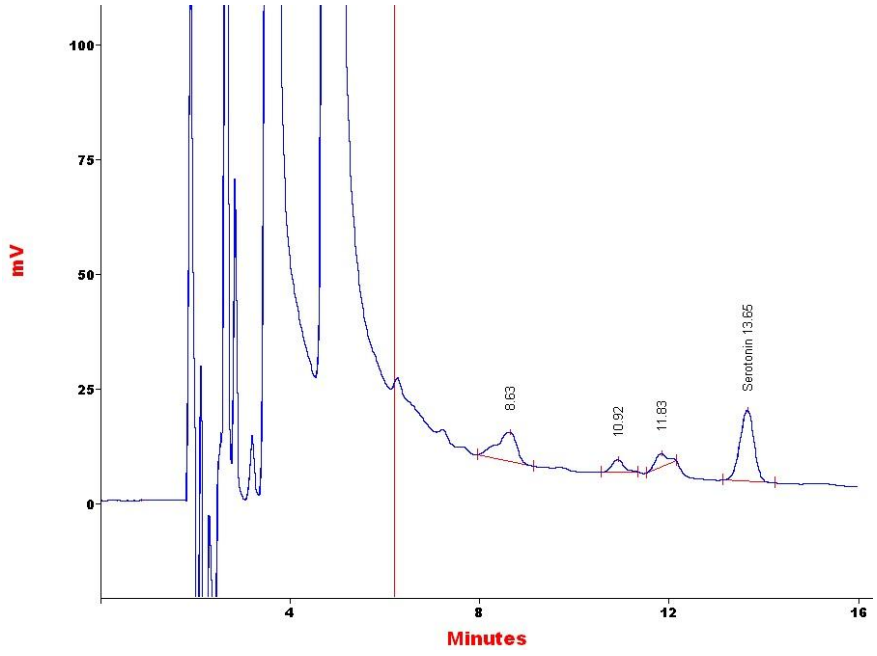


Figure 3.3 Example chromatograms of AADC activity assay samples. Representative chromatogram of a sample of a. L-dopa decarboxylation and b. 5-HTP decarboxylation.

3.4.3 Data Analysis

Dopamine and serotonin were identified and quantified using the AZUR Version 4.6 software package (Datalys) using the following equation:

Conc. (nM) = (sample peak area /external standard peak area) x calibration standard conc. (nM)

The concentration of dopamine or serotonin in the substrate and sample blanks was subtracted from the sample concentration to give the final concentration produced during the incubation period. AADC activity, either L-dopa or 5-HTP decarboxylation, was calculated using the following equation:

AADC activity = (final sample conc. (nM) x dilution factor) / incubation time (min)

These results are expressed as pmol/min/mL. Results for cell homogenates are expressed as pmol/min/mg of protein.

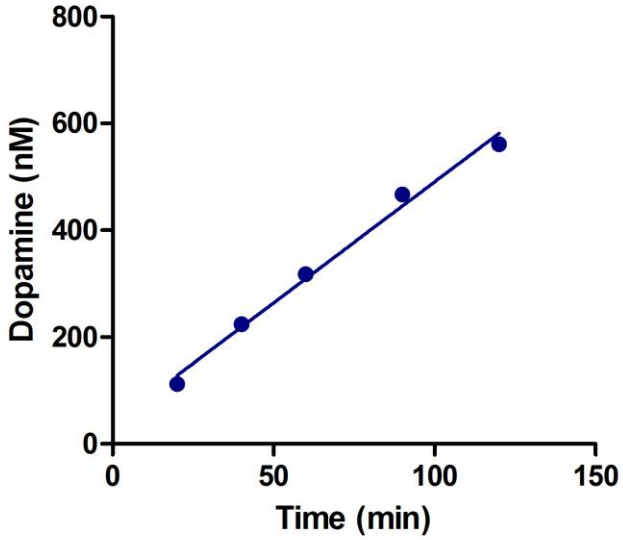
3.5 Validation

3.5.1 Plasma L-dopa decarboxylation

The relationship between incubation time with substrate and dopamine production was determined to be linear across the incubation times tested (20 to 120min; $r^2 = 0.990$; see figure 3.4a). An incubation time of 120min was chosen for future assays to allow adequate dopamine formation during the assay. The relationship between plasma volume and dopamine production was also determined to be linear across tested volumes (10 to 50µL; $r^2 = 0.989$; see figure 3.4b). A plasma volume of 50µL was chosen for future assays. Percentage recovery of dopamine from the reaction mixture containing plasma was 103.2 ± 0.8% (n = 3).

The plasma L-dopa decarboxylation assay was further validated by measuring the kinetics of AADC by varying the concentration of L-dopa added to the reaction between 0.025 and 2mM. L-dopa decarboxylation appeared to follow classical Michaelis-Menten kinetics with the reaction saturated by 1mM L-dopa (see figure 3.5a). K_m apparent and V_{max} were determined using a Lineweaver-Burk plot (Lineweaver and Burk, 1934; see table 1 and figure 3.5b). A concentration of 2mM L-dopa was used for all subsequent assays. The kinetics of the coenzyme PLP was also determined by measuring AADC activity at different concentrations of PLP between 0.5 and 70µM.

a.



b.

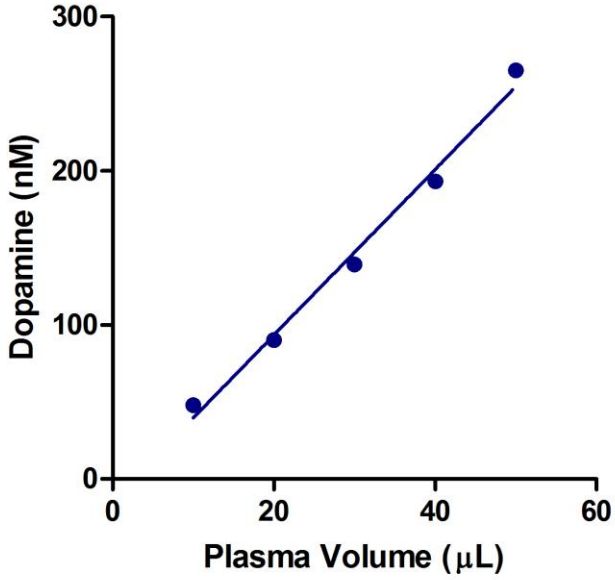


Figure 3.4 Linearity of L-dopa decarboxylation with time and plasma volume. Plasma AADC activity assay using L-dopa as substrate, relationship between a. incubation time and dopamine concentration and b. plasma volume and dopamine concentration.

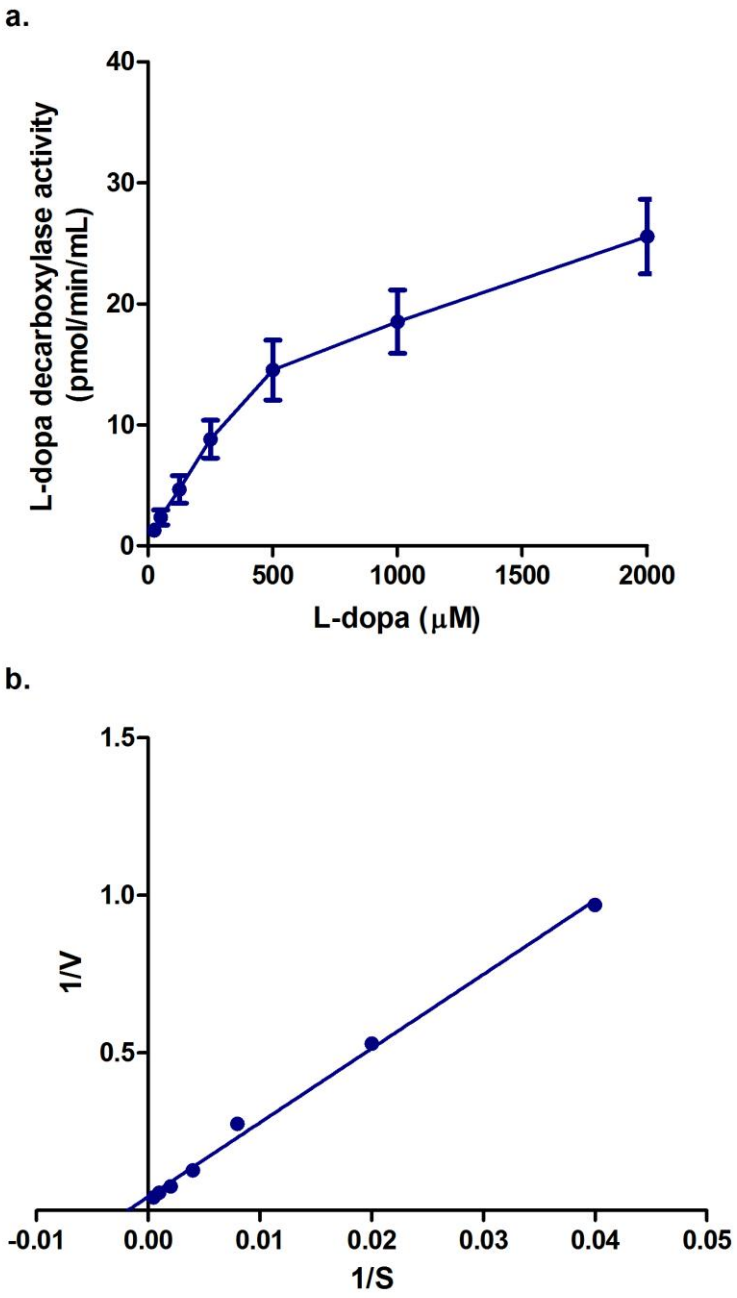


Figure 3.5 Kinetics of AADC activity with L-dopa in plasma. a. Relationship between L-dopa concentration and plasma AADC activity. b. Lineweaver-Burk double-reciprocal plot with L-dopa and plasma AADC activity. 1/V: 1/L-dopa decarboxylase activity (pmol/min/mL), 1/S: 1/L-dopa (μM).

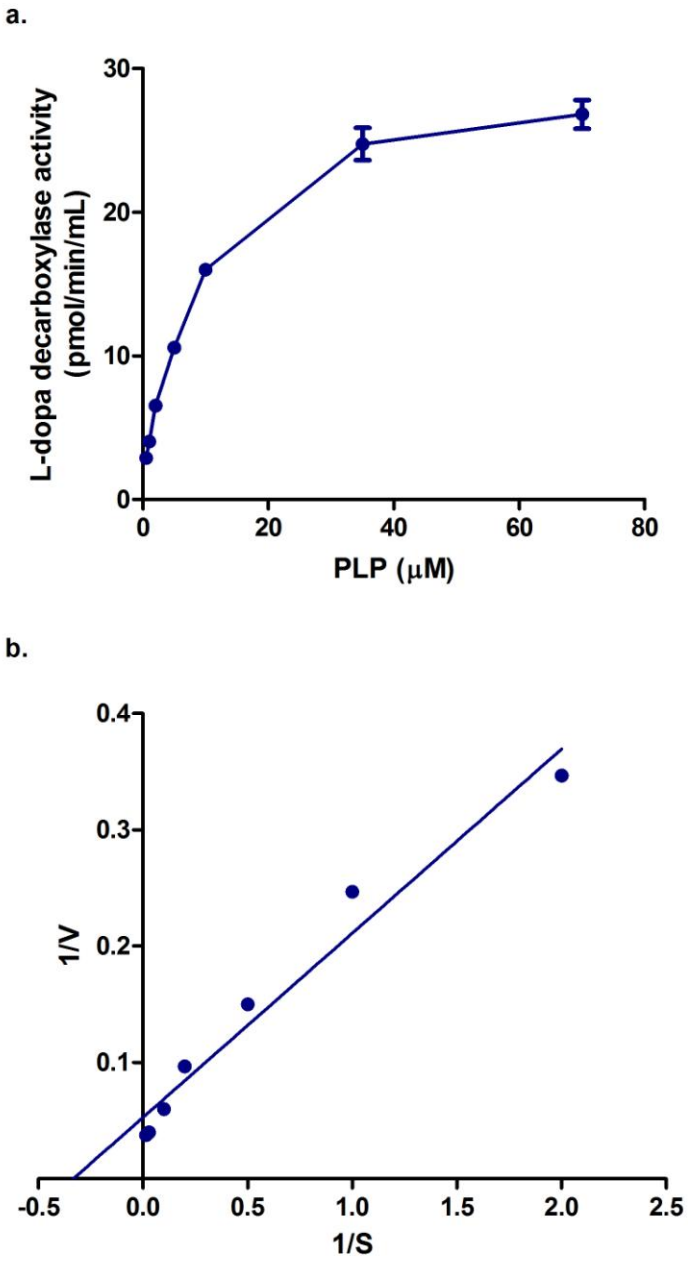


Figure 3.6 Kinetics of AADC activity and PLP in plasma. a. Relationship between PLP concentration and plasma AADC activity with L-dopa as substrate. b. Lineweaver-Burk double-reciprocal plot with PLP and plasma AADC activity with L-dopa as substrate. 1/V: 1/L-dopa decarboxylase activity (pmol/min/mL), 1/S: 1/PLP (μM).

Again the reaction appeared to follow Michaelis-Menten kinetics, saturating by 35μM PLP and K_m apparent and V_{max} were determined by Lineweaver-Burk plot (see figure 3.6 and table 3.1). A concentration of 70μM PLP was used for future assays. Plasma K_m apparent values were similar to those previously reported for L-dopa, 360μM and 620μM, and for PLP, 1.66μM (Boomsma *et al*, 1986; Verbeek *et al*, 2007).

3.5.2 SH-SY5Y cell samples L-dopa decarboxylation

The L-dopa decarboxylation assay was adapted for use with homogenates of the SH-SY5Y cell line. The relationship between dopamine production and incubation time with substrate was found to be linear from 1 to 30min (r^2 = 1.000; see figure 3.7a) but non-linear above 30min. Consequently incubation time was reduced to 5min for all experiments with SH-SY5Y homogenates. The relationship between dopamine production and homogenate total protein was found to be linear across all total protein final concentrations tested from 0.02 to 0.66mg/mL (r^2 = 0.997; see figure 3.7b). For all further experiments with SH-SY5Y homogenates total protein was within this range. Recovery of dopamine from the reaction mixture containing cell homogenate was 105.3 ± 0.7% (n = 3). Both dopamine β-hydroxylase and MAO were likely to be present in SH-SY5Y cell homogenates. Consequently it was tested whether inhibition of these enzymes affected the measurement of L-dopa decarboxylation. Dopamine production after addition of the non-selective MAO inhibitor nialamide (100μM) was 111.3 ± 8.4% of control (p = 0.611, n = 3). Consequently a MAO inhibitor was considered unnecessary and was not used for all subsequent determinations of L-dopa decarboxylation. Dopamine production after the addition of the dopamine β-hydroxylase inhibitor fusaric acid (100μM) was 119.4 ± 1.9% of control (p = 0.366, n = 3). Although the difference from control was not statistically significant it was considered large enough to justify the addition of 100μM fusaric acid to all subsequent assays. The kinetics of AADC was again determined by varying the concentration of L-dopa added to the reaction between 0.025 and 2mM. The reaction followed Michaelis-Menten kinetics, saturating at 1mM L-dopa, and K_m and V_{max} were determined by Lineweaver-Burk plot (see figure 3.8 and table 3.1). A concentration of 2mM L-dopa was used for all future assays. The SH-SY5Y L-dopa K_m apparent was not significantly different from the plasma K_m apparent (p = 0.212) and was within the range of previously reported K_m values in different tissues (120μM to 620μM; Lovenberg *et al*, 1962; Voltattorni *et al*, 1983; Boomsma *et al*, 1986; Moore *et al*, 1996; Jebai *et al*,

1997; Verbeek *et al*, 2007). The kinetics of PLP for L-dopa decarboxyation in SH-SY5Y homogenates could not be determined due to the high proportion of AADC that was already bound to PLP (see below section 3.5.3).

3.5.3 5-HTP decarboxylation cell samples

The 5-HTP decarboxylation assay was adapted for use in SH-SY5Y homogenates from the method using liver homogenates described by Hyland and Clayton (1992). The relationship between serotonin production and incubation time with substrate was tested from 1 to 30min and was found to be linear across this range (r^2 = 0.999; see figure 3.9a). An incubation time of 5min was selected for all further assays. The relationship between serotonin production and homogenate total protein was found to be linear across the range of final protein concentrations tested from 0.02 to 0.33mg/mL (r^2 = 1.000; see figure 3.9b). SH-SY5Y homogenate total protein was within this range for all subsequent experiments. Recovery of serotonin from the reaction mixture containing cell homogenate was 104.9 ± 1.5% (n = 5). It was next determined whether degradation of serotonin by MAO in the cell homogenate would affect the assay. Serotonin production in homogenates treated with 100µM of the non-selective MAO inhibitor nialamide was found to be 102.3 ± 7.4% of control (p = 0.747, n = 3). Consequently the addition of a MAO inhibitor to the reaction mixture was not considered necessary for determination of 5-HTP decarboxylation.

The kinetics of the 5-HTP decarboxylation reaction by AADC was also determined. The concentration of 5-HTP added to the reaction was varied between 5 and 500µM. The reaction followed classical Michaelis-Menten kinetics and saturated by a 5-HTP concentration of 250µM (see figure 3.10a. K_m and V_{max} were determined by Lineweaver-Burk plot (see figure 3.10b; table 3.1). The 5-HTP K_m apparent determined here was within the range of previously reported values from other tissues (20µM to 490µM; Lovenberg *et al*, 1962; Voltattorni *et al*, 1983; Boomsma *et al*, 1986; Moore *et al*, 1996; Jebai *et al*, 1997; Verbeek *et al*, 2007). The proportion of AADC bound to PLP in the samples was estimated by measuring 5-HTP decarboxylation in both the presence and absence of added PLP. Using this method the proportion of AADC bound to PLP was found to be 62 ± 2% (n = 8). The proportion of AADC bound to PLP was not determined using L-dopa decarboxylation as side reactions consuming PLP have previously been demonstrated to lead to underestimation of PLP binding (Kawasaki *et al*, 1992). The proportion of AADC bound to PLP measured by 5-HTP decarboxylation

may also be an underestimation as side reactions consuming PLP could potentially occur during 5-HTP decarboxylation. Nevertheless due to the majority of AADC already being bound to PLP the kinetics of PLP for 5-HTP decarboxylation in SH-SY5Y cell homogenates could not be determined.

Sample	Varied substrate or coenzyme	K_m	V_{max}
Plasma	L-dopa	460.9 ± 96.2	27.1 ± 5.7
Plasma	PLP	3.0 ± 0.4	19.0 ± 1.3
SH-SH5Y	L-dopa	332.6 ± 62.0	4452.6 ± 587.5
SH-SY5Y	5-HTP	47.4 ± 4.9	425.6 ± 22.6

Table 3.1 K_m and V_{max} of AADC in plasma and SH-SY5Y cell samples. K_m (µM) and V_{max} (plasma: pmol/min/mL; SH-SY5Y: pmol/min/mg) values for AADC. The kinetics of PLP was tested using 2mM L-dopa as substrate. Values determined from 3 to 6 independent measurements.

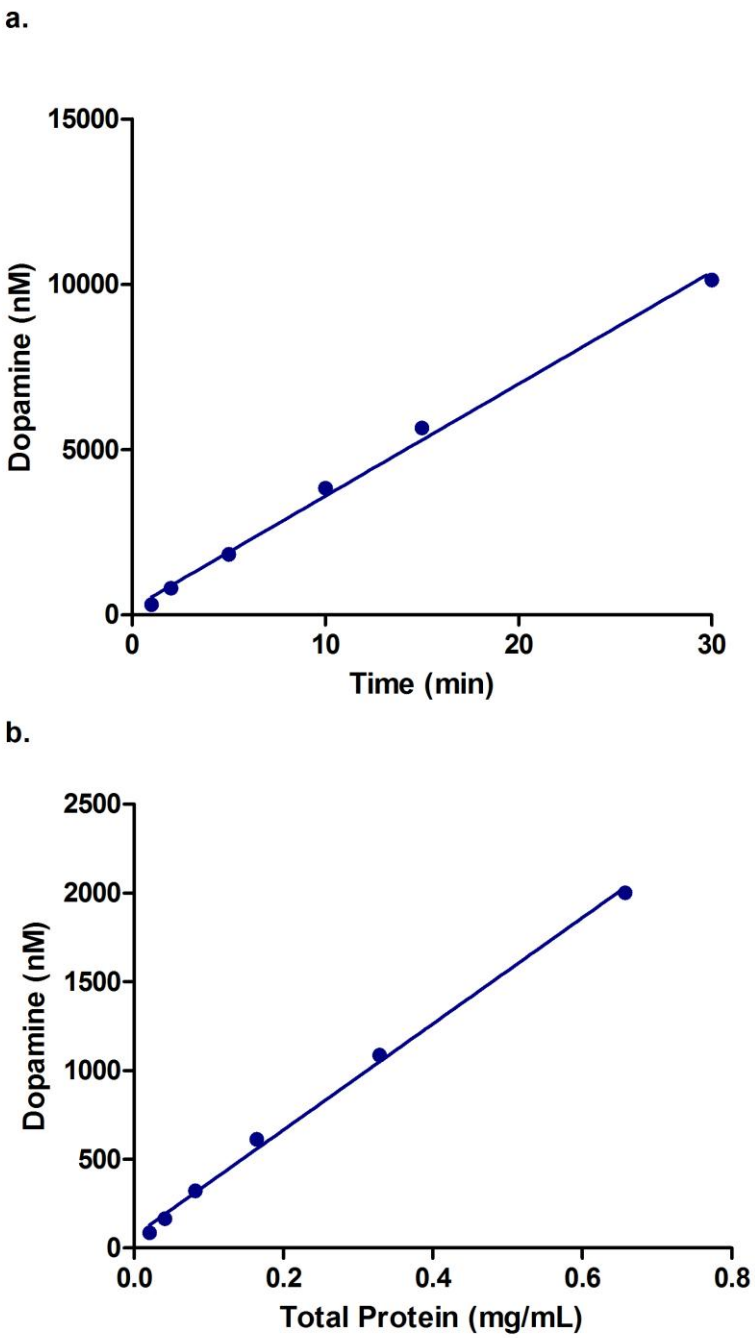


Figure 3.7 Linearity of L-dopa decarboxylation with time and cell homogenate total protein. AADC activity assay in SH-SY5Y homogenate using L-dopa as substrate, relationship between a. incubation time and dopamine concentration and b. plasma volume and dopamine concentration.

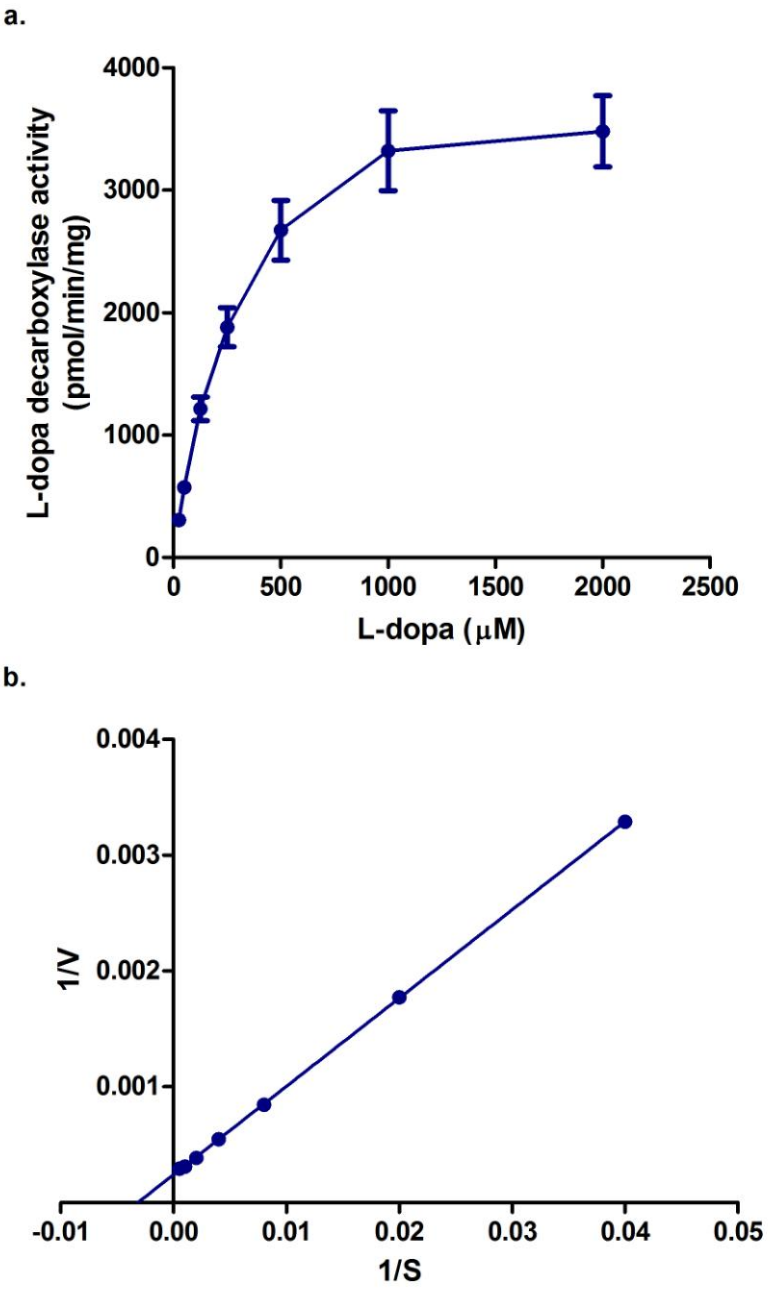


Figure 3.8 Kinetics of AADC activity with L-dopa in SH-SY5Y cell homogenate. a. Relationship between L-dopa concentration and SH-SY5Y homogenate AADC activity. b. Lineweaver-Burk double-reciprocal plot with L-dopa and SH-SY5Y homogenate AADC activity. 1/V: 1/L-dopa decarboxylase activity (pmol/min/mg), 1/S: 1/L-dopa (μM).

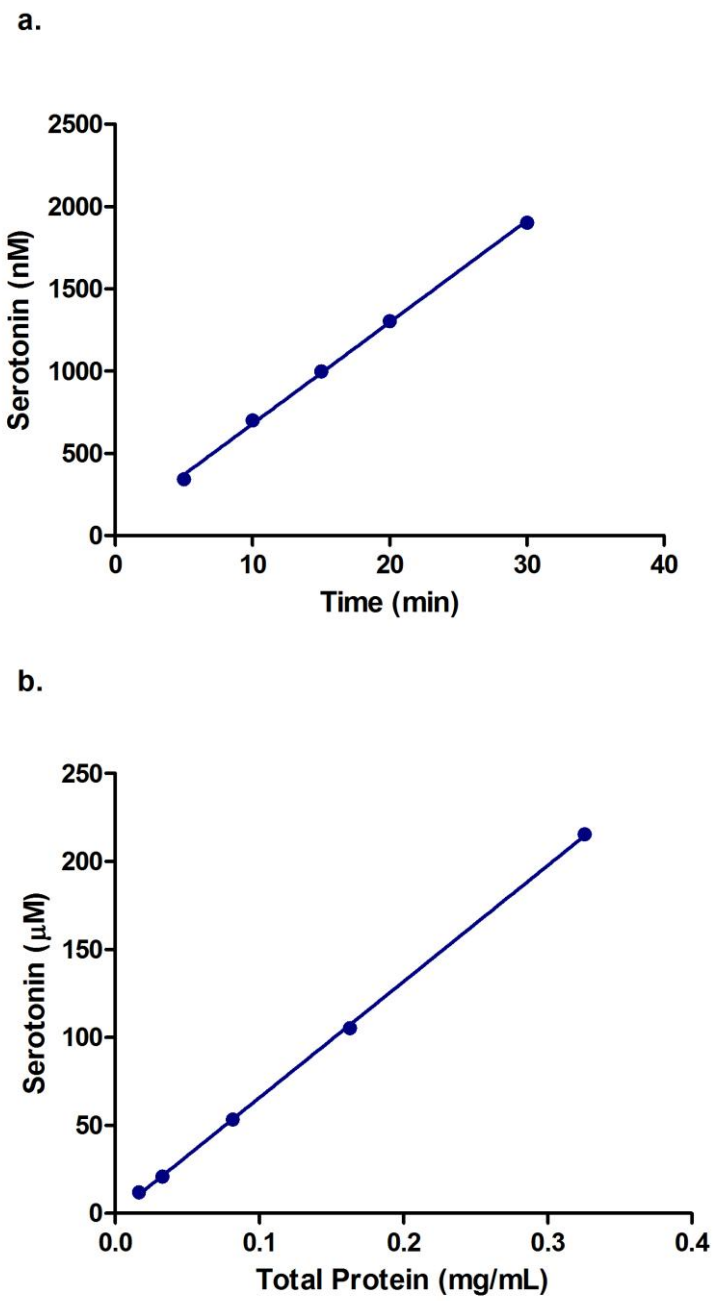


Figure 3.9 Linearity of 5-HTP decarboxylation with time and cell homogenate total protein. SH-SY5Y homogenate AADC activity assay using 5-HTP as substrate, relationship between a. incubation time and serotonin concentration and b. plasma volume and serotonin concentration.

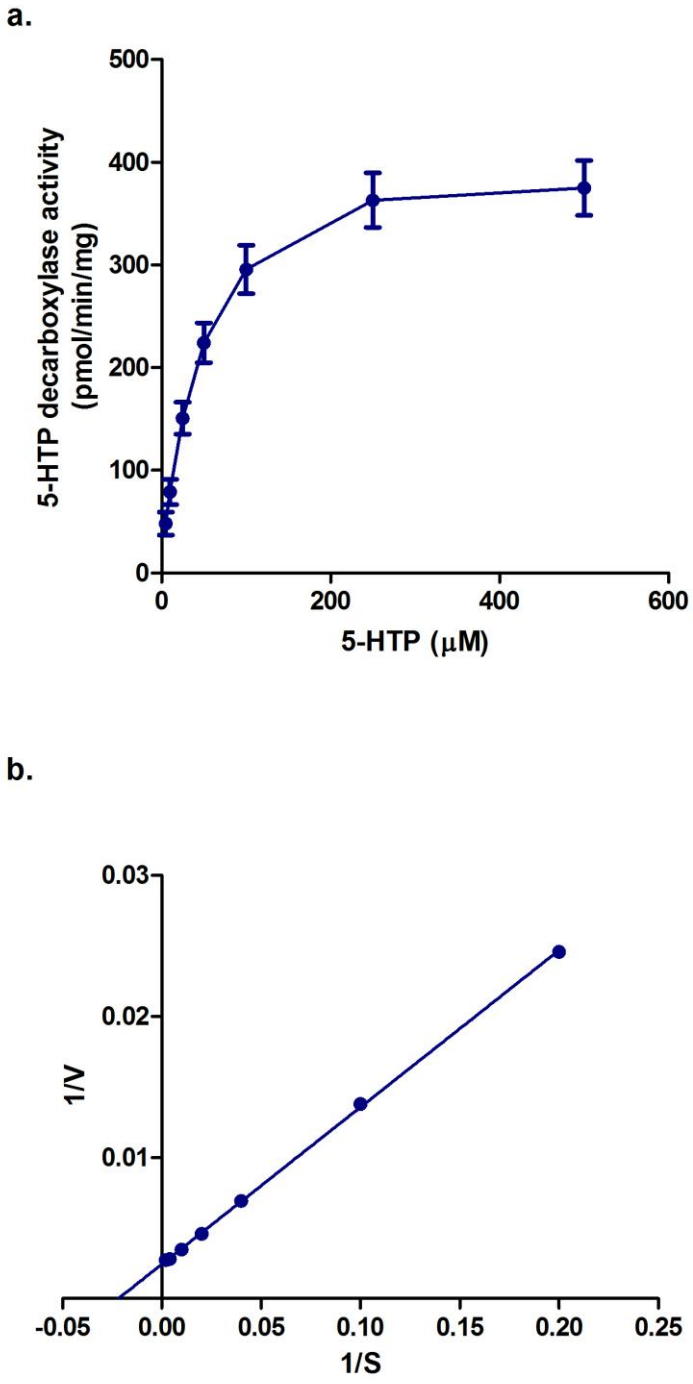


Figure 3.10 Kinetics of AADC activity with 5-HTP in SH-SY5Y cell homogenate. a. Relationship between 5-HTP concentration and SH-SY5Y homogenate AADC activity. b. Lineweaver-Burk double-reciprocal plot with 5-HTP and SH-SY5Y homogenate AADC activity. 1/V: 1/5-HTP decarboxylase activity (pmol/min/mg), 1/S: 1/5-HTP (μM).

Chapter 4

The effect of 5-HTP and serotonin on cell viability, antioxidant status and mitochondrial function

4.1 Introduction

Due to the metabolic block at AADC there is an accumulation of the serotonin precursor 5-HTP in AADC deficient patients including in CSF (Hyland *et al*, 1992; Maller *et al*, 1997; Brun *et al*, 2010). An endogenous accumulation of 5-HTP is relatively unique to AADC deficiency however 5-HTP has been administered in a number of neurological conditions, including disorders of tetrahydrobiopterin metabolism (Ramaekers *et al*, 2001; Turner *et al*, 2006; Longo *et al*, 2009). Furthermore treatment with 5-HTP has been demonstrated to increase the concentrations of 5-HTP present in CSF (Ramaekers *et al*, 2001). 5-HTP administration in animals has been reported to have some side-effects, however these side-effects are commonly considered to relate to increases in serotonin rather than to direct effects of 5-HTP (Colpaert *et al*, 1989; Mokler *et al*, 1992; Gwaltney-Brant *et al*, 2000; Nisijima *et al*, 2001; Turner *et al*, 2006). This "serotonin syndrome" is characterised by a range of behavioural phenotypes including head twitching, flat body posture, tremors and hypolocomotion. These behaviours can be potentiated by serotonin re-uptake inhibitors and MAO inhibitors and prevented with serotonin receptor antagonists, demonstrating the role of increased serotonin availability in these effects (Colpaert *et al*, 1989; Izumi *et al*, 2006). A similar syndrome has been reported in humans after administration of serotonin re-uptake inhibitors (Mills, 1995; Lane and Baldwin, 1997). However this syndrome in humans has not been reported to be induced by 5-HTP administration alone or in combination with serotonin re-uptake inhibitors (Nardini *et al*, 1983; Meltzer *et al*, 1997; Turner *et al*, 2006). Consequently 5-HTP administration is accompanied by relatively few safety concerns (Turner *et al*, 2006), however the direct cellular toxicity of 5-HTP has not been previously reported. Furthermore the toxicity of 5-HTP has not been assessed in the absence of AADC-catalysed production of serotonin.

5-HTP toxicity could potentially arise through alternative metabolism leading to the accumulation of toxic metabolites. A possible alternative metabolic route for 5-HTP could be via the kynurenine pathway (see section 1.7.3). The kynurenine pathway is involved in the *de novo* synthesis of NAD^+ using L-tryptophan as an initial substrate (Beadle *et al*, 1947; Mitchell and Nyc, 1948; Bonner, 1948; Nishizuka and Hayaishi, 1963; Ikeda *et al*, 1965). The first and rate limiting step in this pathway is the conversion of L-tryptophan to *N*-formyl-L-kynurenine (Knox and Mehler, 1950). This

step is catalysed by tryptophan 2,3-dioxygenase in liver and IDO (indoleamine 2,3-dioxygenase) in other tissues (reviewed by Badawy, 1977; Rafice *et al*, 2009). In mouse constitutive IDO expression has been detected in the digestive tract, spleen, thymus and respiratory system (Dai and Zhu, 2010). IDO expression can be induced in response to inflammatory mediators, particularly interferon-γ (IFN-γ), but also interferon-α, interferon-β and lipopolysaccharide (Yoshida *et al*, 1981; Yoshida *et al*, 1986; Takikawa *et al*, 1988; Wang *et al*, 2010a). In the brain IDO expression can be induced by IFN-γ in neurons, astrocytes, microglia and macrophages (Heyes *et al*, 1993; Alberati-Giani *et al*, 1996; Alberati-Giani and Cesura, 1998; Guillemin *et al*, 2003; Guillemin *et al*, 2005b; Kwidzinski *et al*, 2005; Roy *et al*, 2005; Connor *et al*, 2008). Induction of IDO is considered to play a dual role in the immune response. Firstly activation of the rate limiting step of the kynurenine pathway can deplete L-tryptophan, depriving the invading pathogen of this essential amino acid (Byrne *et al*, 1986; Beatty *et al*, 1994). Secondly IDO induction can suppress T-cell proliferation and consequently acts to limit the immune response, potentially preventing damage to uninfected tissue (Munn *et al*, 1999; Hwu *et al*, 2000). This latter function is also mediated by L-tryptophan depletion, but additionally by production of pro-apoptotic kynurenine metabolites (Munn *et al*, 1999; Hwu *et al*, 2000; Morita *et al*, 2001). Several kynurenine metabolites of L-tryptophan are also known to have neuroactive properties including the putative neurotoxins quinolinic acid and 3-hydroxykynurenine (see section 1.7.3; Foster *et al*, 1983; Garthwaite and Garthwaite, 1987; Okuda *et al*, 1998; Guidetti and Schwarcz, 1999; Santamaria *et al*, 2001). These kynurenine metabolites have been implicated in Huntington's disease, dementia associated with HIV infection and ischaemic brain damage (Moroni *et al*, 1999; Guidetti and Schwarcz, 2003; Guillemin *et al*, 2005c; Samikkannu *et al*, 2010; Sathyasaikumar *et al*, 2010).

5-HTP is also known to be a substrate for IDO (Hirata and Hayaishi, 1972; Shimizu *et al*, 1978; Fujiwara *et al*, 1979). Human IDO purified from placenta had a K_m for 5-HTP of 400μM, whilst the K_m for L-tryptophan was 21μM, indicating that L-tyrptophan is likely to be the preferred substrate (Takikawa *et al*, 1988). However the 5-HTP-kynurenine pathway products 5-hydroxykynurenine and 4,6-dihydroxyquinoline (see figure 1.6) have been detected in the urine of hens after an L-tryptophan load, demonstrating that 5-HTP-kynruenine metabolites can be produced *in vivo* (Kido *et al*, 1967a Kido *et al*, 1967b). Furthermore the conversion of 5-HTP to 5-hydroxyformylkynurenine and subsequently 5-hydroxykynruenine has been suggested

to occur in rat brain homogenates and consequently the CNS may also produce 5-HTP-kynruenine metabolites (Tsuda *et al*, 1972; Tsuda *et al*, 1974). Very little has been reported as to the physiological effects or functions of the 5-HTP-kynurenine pathway metabolites. 5-hydroxykynuramine, which can be produced from 5-HTP or directly from serotonin, has been demonstrated to interact with serotonin receptors (Watts *et al*, 1995). This metabolite can induce contraction of smooth muscle and increase prolactin release although with less potency than serotonin (Toda *et al*, 1974; Toda, 1975; Iwasaki *et al*, 1978; Kitamura *et al*, 1979; Fu and Toda, 1979). 5-hydroxykynuramine has also been shown to antagonise serotonin induced platelet aggregation (Okuma *et al*, 1976). 5-hydroxyanthranillic acid is a potential 5-HTP kynurenine metabolite that can also be produced from L-tyrptophan metabolism via the kynurenine pathway (Noguchi *et al*, 1969; Naito *et al*, 1984). It has recently been demonstrated that 5-hydroxyanthranillic acid can induce oxidative stress, H_2O_2 production and neuronal cell death in culture (Smith *et al*, 2009). The accumulation of 5-HTP in AADC deficiency may potentially also lead to the accumulation of 5-HTP-kynurenine metabolites. These kynurenine metabolites or 5-HTP itself could potentially induce oxidative stress. Two potential consequences of oxidative stress could be decreases in the level of neuronal GSH and decreased activity of the mitochondrial respiratory chain (see section 1.7.6 and section 1.7.7; Bolanos *et al*, 1994; Desole *et al*, 1995; Heales *et al*, 1995; Bolanos *et al*, 1996; Merad-Boudia *et al*, 1998; Merad-Saidoune *et al*, 1999; Chinta and Andersen, 2006). Consequently it may be of importance for understanding the pathogenesis of AADC deficiency to investigate the role played by the kynurenine pathway when 5-HTP availability is increased.

4.2 Aims

1) To determine the toxicity of 5-HTP and serotonin in neuronal and astrocyte cell lines

2) To examine the effect of IFN-γ on 5-HTP and serotonin toxicity and metabolism

3) To determine the effect of increased levels of 5-HTP and serotonin on intracellular GSH and mitochondrial respiratory chain activity in a neuronal cell line

4.3 Methods

4.3.1 Treatment solutions

20mM NSD-1015, 40mM 5-HTP, 40mM serotonin were made up as stock solutions in HPLC grade H_2O, 0.2µm sterile filtered and stored at -20˚C. Sterile IFN-γ was reconstituted to 2000U/mL in DMEM/F-12 + 100mL/L FBS and stored at -80°C in 1mL aliquots.

4.3.2 Cell culture

Routine cell culture was performed as described in section 2.2.6 for SH-SY5Y cells and in section 2.2.7 for 1321N1 cells.

4.3.3 Measurement of Indoleamines

Cell homogenates and cell culture medium were prepared as described in section 2.2.8.1 and indoleamines were measured as described in section 2.2.3.

4.3.4 AADC activity

Cell homogenates were prepared as described in section 3.3.2 and AADC activity was measured as described in section 3.3.3.

4.3.5 Western Blotting

Cells were prepared as described in section 2.2.8.4 and western blot was performed as described in section 2.5.

4.3.6 RT-PCR

RNA was isolated as decribed in section 2.6.2. RT-PCR was performed as described in section 2.6.3 and agarose gel electrophoresis was performed as described in section 2.6.4.

4.3.7 Measurement of GSH

Cells and minimal medium were prepared as described in section 2.2.8.2 and section 2.2.8.3 and GSH measured as described in section 2.2.4.

4.3.8 SRB cell proliferation assay

The SRB cell proliferation assay was performed as described in section 2.3.

4.3.9 LDH Release assay

The LDH release assay was performed as described in section 2.4

4.3.10 Mitochondrial respiratory chain complex assays and citrate synthase

The activity of complex I, II/III, IV and citrate synthase were determined by the methods described in section 2.7

4.4 Experimental Protocol

4.4.1 Indoleamine measurement

SH-SY5Y cells were seeded in 12.5mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 56.7cm^2 cell culture dishes. 6.25µL NSD-1015 (20mM: final conc. 10µM; 2mM: final conc. 1µM; 0.2mM: final conc. 0.1µM) and/or 312.5µL 2000U/mL IFN-γ (final conc. 50U/mL) were added to appropriate dishes and 6.25µL sterile HPLC grade H_2O was added to untreated dishes. Cells were incubated for 24hr at 37˚C in 5.0% CO_2 to allow cells to adhere. 15.63µL 5-HTP (40mM: final conc. 50µM; 8mM: final conc. 10µM) or 40mM serotonin (final conc. 50µM) were added to appropriate dishes and cells incubated for 48hr at +37˚C in 5.0% CO_2. Cells and cell culture medium were then processed as indicated in section 4.3.3.

4.4.2 Toxicity testing

For SRB and LDH release assays SH-SY5Y cells or 1321N1 cells were seeded in 96-well plates at a density of 1.80 x 10^4 cells/well in DMEM/F-12 + 100mL/L FBS or DMEM + 100mL/L FBS respectively. 10µL NSD-1015 (200µM: final conc. 10µM or 20µM: final conc. 1µM) was added to treated wells, 10µL sterile HPLC grade H_2O was added to untreated wells. 10µL 1000U/mL IFN-γ (final conc. 50U/mL) was added to wells where indicated. Plates were incubated for 24hr to allow cells to adhere at +37˚C in 5.0% CO_2. 10µL 5-HTP or serotonin (concentration range: 40mM to 2mM: final conc. 2000µM to 100µM) were added to appropriate treated wells. 10µL sterile HPLC grade H_2O was added to control wells. Final volume of all wells was 200µL. Cells were then incubated for 48hr at +37˚C in 5.0% CO_2. Cells were then processed as indicated for the appropriate assay (see section 4.3.8 for the SRB assay and section 4.3.9 for LDH release assay).

4.4.3 GSH measurement

SH-SY5Y cells were seeded in 3mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 6-well cell culture dishes, the surface area of each well was 9.6cm^2. 30µL 1mM NSD-1015 (final conc. 10µM) and/or 75µL 2000U/mL IFN-γ were added to appropriate treated wells. 30µL sterile HPLC grade H_2O was added to untreated wells. Plates were incubated at +37˚C in 5.0% CO_2 for 24hr to allow cells to adhere. 75µL 5-HTP or serotonin (40mM: final conc. 1mM; 2mM: final conc. 50µM) were added to appropriate treated wells. 75µL sterile HPLC grade H_2O was added to untreated wells. Plates were then incubated at +37˚C in 5.0% CO_2 for the indicated length of time. Cells were processed as indicated for determination of intracellular GSH (see section 4.3.7).

4.4.4 Mitochondrial respiratory chain and citrate synthase

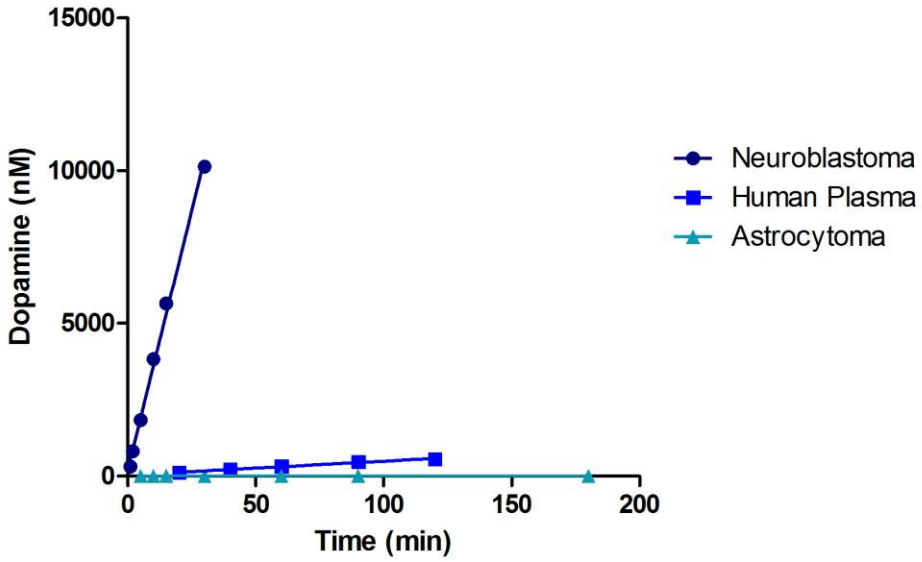
SH-SY5Y cells were seeded in 30mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 145cm^2 cell culture dishes. 15µL 20mM NSD-1015 (final conc. 10µM) was added to treated dishes and 15µL sterile HPLC grade H_2O was added to untreated dishes. Cells were incubated for 24hr at +37˚C in 5.0% CO_2 to allow cells to adhere. 37.5µL 40mM 5-HTP (final conc. 50µM) or 40mM serotonin (final conc. 50µM) were added to appropriate dishes, 37.5µL sterile HPLC grade H_2O was added to control dishes. Cells were incubated for 48hr at +37˚C in 5.0% CO_2. Cells were then processed as indicated for respiratory chain complex assays (see section 4.3.10).

4.5 Results

4.5.1 AADC in SH-SY5Y and 1321N1 cells

The activity of AADC in SH-SY5Y cells and 1321N1 cells was first determined. The AADC activity with L-dopa as substrate was found to be 3462.7 ± 319.2 pmol/min/mg for SH-SY5Y cells, whilst in 1321N1 cells activity was below the limit of detection (n = 7). This result indicates that 1321N1 cells do not contain active AADC. As a further test 1321N1 cell homogenate was incubated for up to 3hr with L-dopa without any detectable increase in dopamine (see figure 4.1a). To confirm that the AADC activity of SH-SY5Y cells was related to AADC expression both AADC mRNA and protein were determined by RT-PCR and western blot respectively (see figure 4.1b).

a.



b.

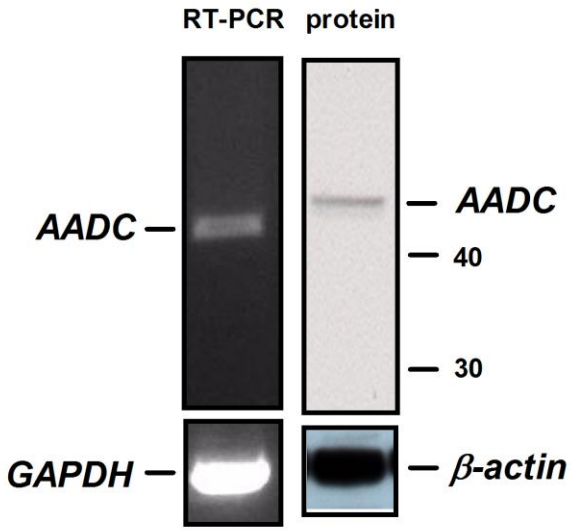


Figure 4.1 AADC activity, mRNA and protein in cells. a. The AADC activity of SH-SY5Y neuroblastoma cells, human plasma and 1321N1 astrocytoma cells plotted as incubation time with L-dopa against dopamine. b. SH-SY5Y cells expression of AADC mRNA and protein. Representative images from 3 independent experiments.

4.5.2 NSD-1015 concentration for inhibition of AADC

The appropriate concentration range for inhibition of AADC activity in SH-SY5Y cells was determined. Cells were incubated with NSD-1015 from 0 to 10μM for 24hr. 10μM 5-HTP was added and cells were incubated for 48hr before measurement of indoleamines in the cell culture medium. Serotonin and 5-HIAA were significantly decreased and 5-HTP was significantly increased following treatment with 1μM or 10μM NSD-1015 in comparison to control (see figure 4.2). This indicates that AADC was inhibited at these concentrations with the greater inhibition seen at 10μM. 0.1μM NSD-1015 treatment had no significant effect upon 5-HIAA, serotonin or 5-HTP in comparison to control. Concentrations of 1μM or 10μM NSD-1015 were chosen for all future experiments. For further investigations of indoleamine metabolism the concentration of 5-HTP was increased to 50μM in order to increase the availability of substrate during the incubation.

4.5.3 Indoleamine metabolism

SH-SY5Y cells were cultured either in the presence or absence of 10μM NSD-1015 for 24hr and then subsequently 50μM 5-HTP was added for 48hr. Treatment with NSD-1015 led to a significant decrease in intracellular 5-HIAA and extracellular serotonin and 5-HIAA in comparison to untreated control (see table 4.1). Furthermore 5-HTP was increased both intracellularly and in the cell culture media, indicating that AADC was partially inhibited, however intracellular serotonin was not significantly affected by NSD-1015 treatment. IFN-γ was used throughout these experiments to potentially activate IDO and consequently the kynurenine pathway. A concentration of 50U/mL IFN-γ was chosen for experiments based on previous investigations (Martin *et al*, 1993; Chiarugi *et al*, 2000). In order to assess the effect of IFN-γ stimulation on indoleamine metabolism SH-SY5Y cells were cultured in the presence of IFN-γ with or without 10μM NSD-1015 and indoleamines were measured following a 48hr incubation with 50μM 5-HTP. IFN-γ treatment led to a 1.4-fold increase in the intracellular level of 5-HIAA in comparison to untreated control ($p < 0.05$). However this effect of IFN-γ was not observed in NSD-1015 treated cells in comparison to those treated with NSD-1015 only. Conversely in NSD-1015 treated cells intracellular levels of 5-HTP were increased by approximately 1.6-fold ($p < 0.01$) following IFN-γ treatment in comparison to cells treated with NSD-1015 alone. This effect was not observed in cells treated with IFN-γ alone in comparison to untreated control. Intracellular serotonin and extracellular

5-HTP, serotonin and 5-HIAA were unaffected by IFN-γ treatment both in the presence and absence of NSD-1015. Following treatment with 50µM serotonin for 48hr IFN-γ treatment did not affect the intracellular or extracellular levels of 5-HTP, serotonin or 5-HIAA in comparison to cells treated with serotonin alone

4.5.4 5-HTP and serotonin toxicity in SH-SY5Y cells

The SRB cell proliferation assay was utilised to investigate the effect of 5-HTP and serotonin on SH-SY5Y cell growth. Treatment for 48hr with 5-HTP at concentrations >500µM and serotonin at concentrations >1000µM led to significant reductions in cell growth (see figure 4.3a). Comparing the effect on cell growth of 5-HTP to that of serotonin there was an overall difference ($p < 0.001$), however by individual comparison this difference was only significant at 250µM ($p < 0.05$). The addition of 1µM and 10µM NSD-1015 did not have an effect on the decrease in cell growth seen with 5-HTP treatment alone ($p = 0.735$; see figure 4.3b). The effect of 5-HTP and serotonin on membrane integrity was investigated using the LDH release assay. 5-HTP treatment for 48hr led to a small but significant increase in LDH release at concentrations >1000µM (see figure 4.4a). 48hr treatment with 2000µM serotonin significantly increased LDH release by approximately 2-fold above the level of untreated control ($p < 0.05$). Concentrations of serotonin below 2000µM had no effect on LDH release. The overall effect of 5-HTP and serotonin on LDH release was not significantly different ($p < 0.107$), however by individual comparison the effect of serotonin on LDH release was significantly different from that of 5-HTP at 2000µM ($p < 0.01$). NSD-1015 at a concentration of 1µM or 10µM had no significant effect on LDH release observed following 5-HTP treatment ($p = 0.234$; see figure 4.4b).

As an activator of IDO the influence of IFN-γ on 5-HTP and serotonin treatment of SH-SY5Y cells was also investigated. Cells were treated with 50U/mL IFN-γ for 24hr prior to the addition of 5-HTP and then incubated for 48hr. Using the SRB assay IFN-γ treatment alone decreased cell growth to approximately 37% of untreated control ($p < 0.001$). 5-HTP was found to significantly affect cell growth of IFN-γ treated cells at concentrations >500µM (see figure 4.5a). At concentrations >1000µM 5-HTP treatment led to some cell loss relative to the amount of cells present at time 0. The effect of 5-HTP on cell growth in the presence and absence of IFN-γ was found to be significantly different ($p < 0.001$). Cell growth following treatment with 1µM NSD-1015 was less affected by 5-HTP ($p < 0.01$) however this effect was not significant with

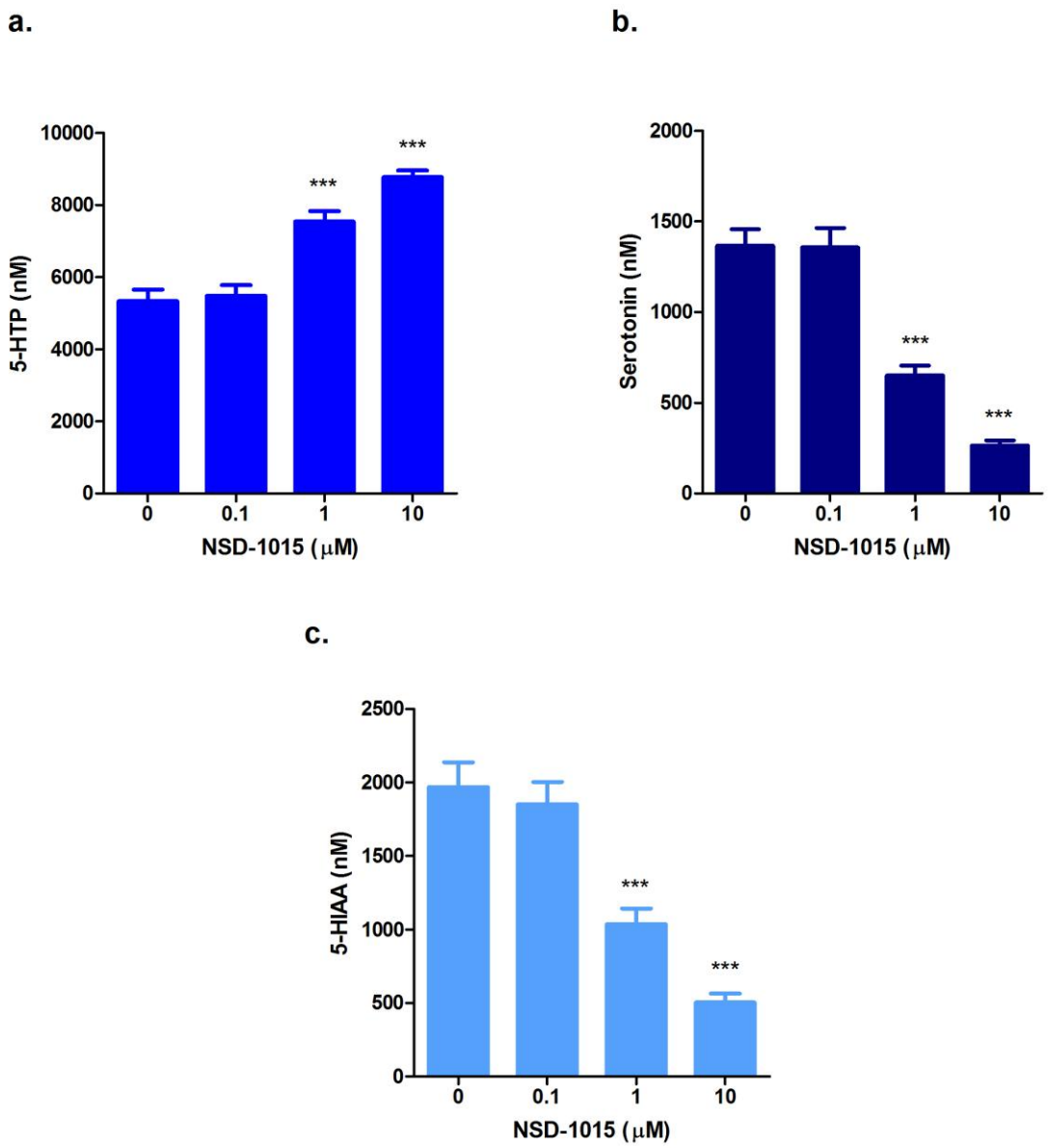


Figure 4.2 NSD-1015 concentration for inhibition of AADC in SH-SY5Y cells. Extracellular concentration of a. 5-HTP b. serotonin and c. 5-HIAA in SH-SY5Y cells following 48hr treatment with 10μM 5-HTP in the presence of different concentrations of NSD-1015. All values are mean ± SEM of 8 independent experiments . *** $p < 0.001$ in comparison to 5-HTP alone treatment determined by ANOVA followed by least significant difference test.

10µM NSD-1015 ($p = 0.120$). Serotonin treatment in the presence of IFN-γ also had a significant effect on SH-SY5Y cell growth at concentrations >500µM (see figure 4.5b). Cell loss relative to the amount of cells present before treatment was observed at concentrations >750µM. The effect of serotonin on cell growth in the presence of IFN-γ was found to be different from the effect of serotonin alone ($p < 0.001$). Furthermore in the presence of IFN-γ serotonin treatment induced cell loss at lower concentrations (750µM) than 5-HTP (1000µM; $p < 0.05$).

IFN-γ treatment alone increased LDH release from SH-SY5Y cells by approximately 1.4-fold in comparison to untreated control ($p < 0.01$). In the presence of IFN-γ 5-HTP had no significant effect on LDH release at concentrations <2000µM (see figure 4.6a). Comparing cells treated with 5-HTP alone to those treated with 5-HTP and IFN-γ LDH release was increased ($p < 0.001$). 1µM or 10µM NSD-1015 treatment did not alter LDH release after treatment with <2000µM 5-HTP in the presence of IFN-γ ($p = 0.386$; see figure 4.6a). Serotonin treatment in combination with IFN-γ led to significant increases in LDH release at serotonin concentrations >1000µM in comparison to control treated with IFN-γ alone (see figure 4.6b). The effect of 1000µM and 2000µM serotonin upon LDH release was significantly increased in the presence of IFN-γ in comparison to SH-SY5Y cells treated with serotonin alone. The effect of serotonin in the presence of IFN-γ was also found to be increased in comparison to 5-HTP in the presence of IFN-γ ($p < 0.01$).

4.5.5 Effect of 5-HTP and serotonin on LDH release in astrocytoma cells

The LDH release assay was used to investigate the effect of 5-HTP and serotonin on membrane integrity of the human astrocytoma cell line 1321N1. Cells were treated with 5-HTP or serotonin for 48hr before measurement of LDH release. 5-HTP treatment did not change LDH release across the concentration range tested ($p = 0.720$; see figure 4.7). 2000µM serotonin treatment led to an approximately 3.3-fold increase in LDH release ($p < 0.05$) with no significant effect observed at lower concentrations of serotonin (see figure 4.7). The effect of IFN-γ upon LDH release from 1321N1 cells was also investigated. Cells were treated for 24hr with IFN-γ before the addition of 5-HTP or serotonin. IFN-γ treatment alone had no effect on LDH release ($p = 0.763$). In the presence of IFN-γ 5-HTP at a concentration of 1000µM increased LDH release by

TREATMENT	5-HTP	Serotonin	5-HIAA
INTRACELLULAR			
5-HTP	1 ± 0	1584 ± 198	281 ± 21
5-HTP + IFNγ	9 ± 1	2244 ± 344	399 ± 54*
5-HTP + NSD-1015	194 ± 11***	1278 ± 155	39 ± 8***
5-HTP + NSD-1015 +IFN-γ	308 ± 46***++	1508 ± 180	24 ± 4***
serotonin	ND	1868 ± 238	291 ±37
Serotonin + IFN-γ	ND	1911 ± 208	326 ± 64
EXTRACELLULAR			
5-HTP	5968 ± 350	2280 ± 78	1928 ± 336
5-HTP + IFNγ	5387 ± 505	2422 ± 99	1578 ± 280
5-HTP + NSD-1015	10840 ± 199**	188 ± 5***	205 ± 39***
5-HTP + NSD-1015 +IFN-γ	10470 ± 331***	185 ± 2***	156 ± 36***
serotonin	ND	9361 ± 396	1910 ± 405
Serotonin + IFN-γ	1.62 ± 1.62	9763 ± 499	2075 ± 335

Table 4.1 Effect of NSD-1015 and IFN-γ on indoleamine metabolism. Indoleamine levels following treatment with 50µM 5-HTP or Serotonin. Cells treated with 10µM NSD-1015 and/or 50U/mL IFN-γ for 24hr before the addition of 5-HTP or serotonin. Intracellular values expressed as pmol/mg of protein and extracellular values expressed as nM. All values are mean ± SEM of 5 independent experiments. ND: not detectable. $^{++}$ $p < 0.01$ in comparison to 5-HTP + NSD-1015 treatment, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ in comparison to 5-HTP alone treatment determined by ANOVA followed by least significant difference test.

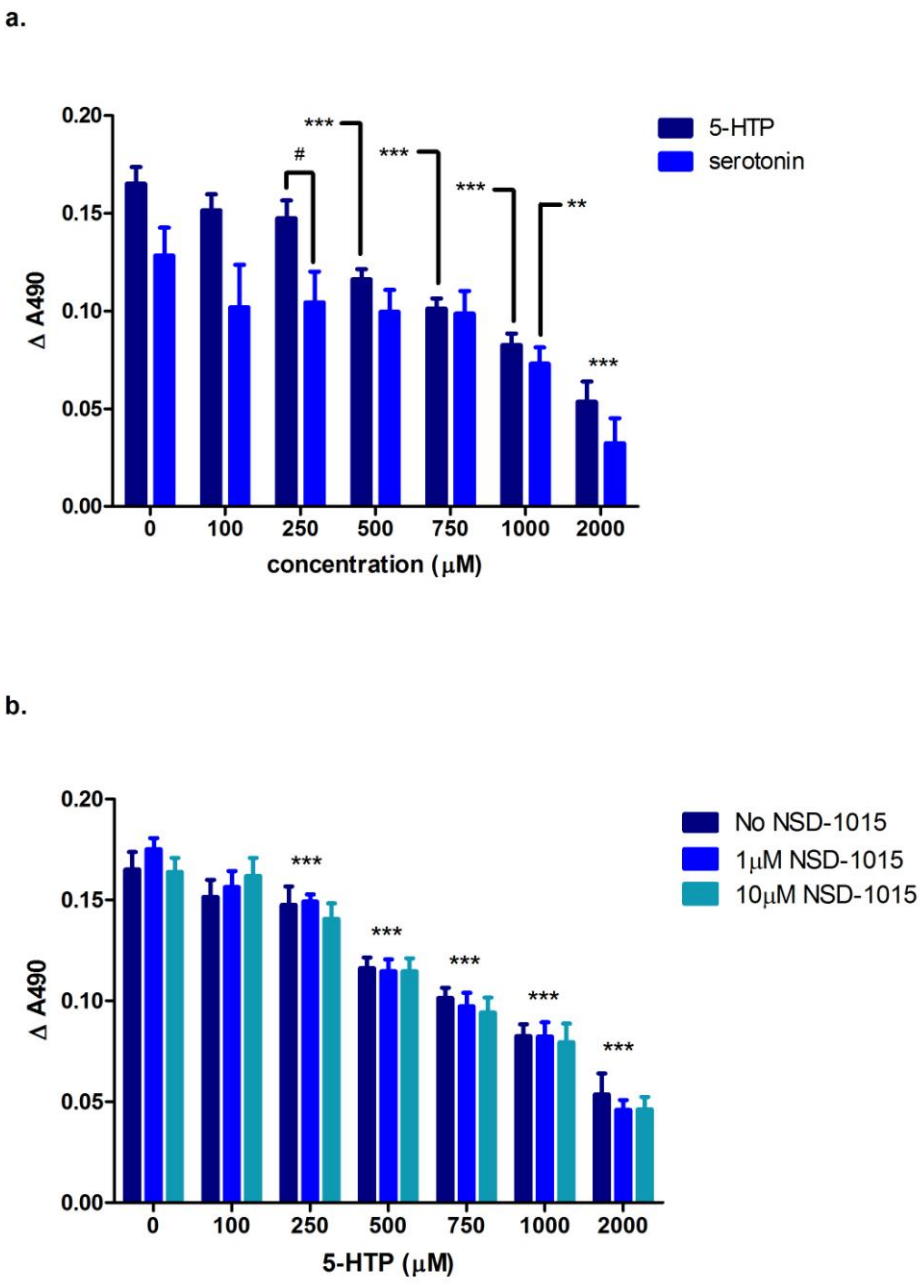


Figure 4.3 SH-SY5Y cell proliferation following 5-HTP, serotonin and NSD-1015 treatment. a. The effect of 5-HTP and serotonin treatment on cell proliferation of SH-SY5Y cells as measured by SRB assay. b. The effect of NSD-1015 on 5-HTP induced changes in cell proliferation of SH-SY5Y cells measured by SRB assay. ΔA490: Change in absorbance at 490nm compared to cells prior to treatment. Results are mean ± SEM of 6 independent experiments. ** $p < 0.01$, ***$p < 0.001$ difference in comparison to 0 by ANOVA followed by least significant difference test. $^{\#}$ $p < 0.05$ determined by Student's t-test.

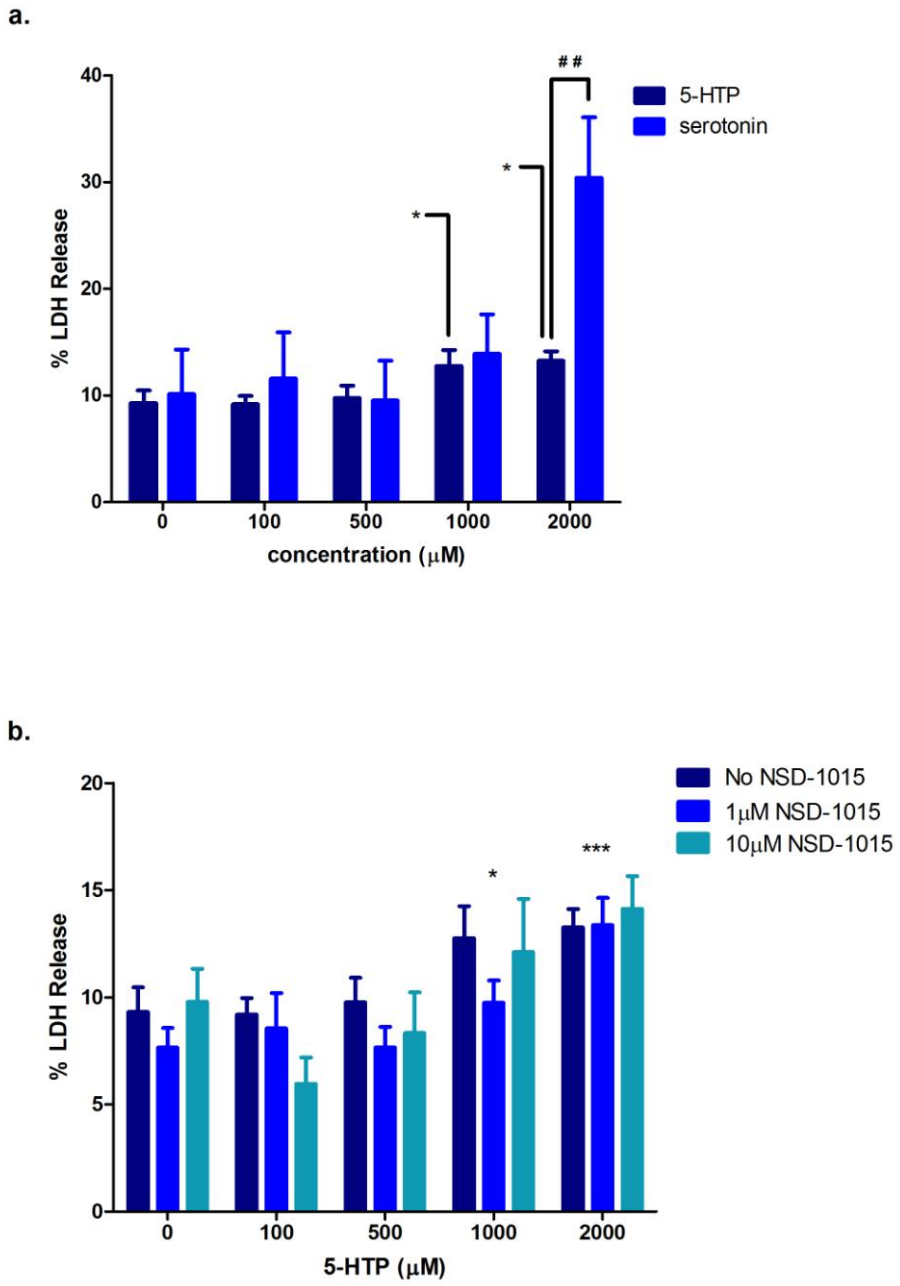


Figure 4.4 SH-SY5Y LDH release following 5-HTP, serotonin and NSD-1015 treatment. a. The effect of 5-HTP and serotonin on membrane integrity of SH-SY5Y cells measured by the LDH release assay. b. LDH release from SH-SY5Y cells in response to 5-HTP following treatment with NSD-1015. % LDH release: LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis. Results are mean ± SEM of 5 independent experiments. * $p < 0.05$, ***$p < 0.001$ difference in comparison to 0 by ANOVA followed by least significant difference test. [#] [#] $p < 0.01$ determined by Student's t-test.

approximately 1.3-fold in comparison to cells treated with IFN-γ alone ($p < 0.01$; see figure 4.8a), however this effect was not significantly different from treatment with 5-HTP alone ($p = 0.09$). Higher and lower concentrations of 5-HTP had no significant effect on LDH release in the presence of IFN-γ. IFN-γ also had no significant effect upon LDH release from 1321N1 cells treated with serotonin in comparison to cells treated with serotonin alone ($p = 0.880$; see figure 4.8b).

4.5.6 Intracellular GSH in SH-SY5Y cells

To investigate whether the effects of 5-HTP on cell growth were related to oxidative stress intracellular GSH in SH-SY5Y cells was measured. Cells were incubated in the presence or absence of IFN-γ and/or 10μM NSD-1015 for 24hr before the addition of 5-HTP. IFN-γ and NSD-1015 ($p = 0.509$ and $p = 0.159$ respectively) had no significant effect on intracellular GSH with 5-HTP treatment (see figure 4.9a). 5-HTP treatment led to a small but significant increase in GSH concentration at both 50μM and 1000μM ($p < 0.05$, $p < 0.01$ respectively), although comparing these two concentrations this effect did not appear to be dose-dependent ($p = 0.523$). Similarly small increases in intracellular GSH with both 50μM and 1000μM of serotonin were observed ($p < 0.05$, $p < 0.01$ respectively; see figure 4.9b) again with no significant difference between the two concentrations ($p = 0.378$). IFN-γ had no effect upon the changes in GSH levels following serotonin treatment ($p = 0.244$).

4.5.7 Mitochondrial respiratory chain activity following 5-HTP or serotonin treatment

As GSH was increased with 5-HTP and serotonin treatment at a sub-toxic concentration of 50μM this indicates that there may be cellular alterations at lower and potentially more physiologically relevant concentrations. Furthermore alterations in GSH levels can lead to changes in the activity of the mitochondrial respiratory chain complexes (Heales *et al*, 1995). The effect of 50μM 5-HTP and serotonin on the mitochondrial respiratory chain was therefore also investigated. 50μM 5-HTP or serotonin alone produced no change in complex I, II/III or IV activity in comparison to untreated control (see figure 4.10 and table 4.2). Similarly 50μM 5-HTP in the presence of NSD-1015 did not alter the activity of any of the complexes in comparison to cells treated with NSD-1015 alone. Treatment with 5-HTP and IFN-γ did result in an increase in complex II activity in comparison to cells treated with 5-HTP alone (see figure 4.10 and table 4.2). This effect was apparent both when activity was expressed as a ratio to

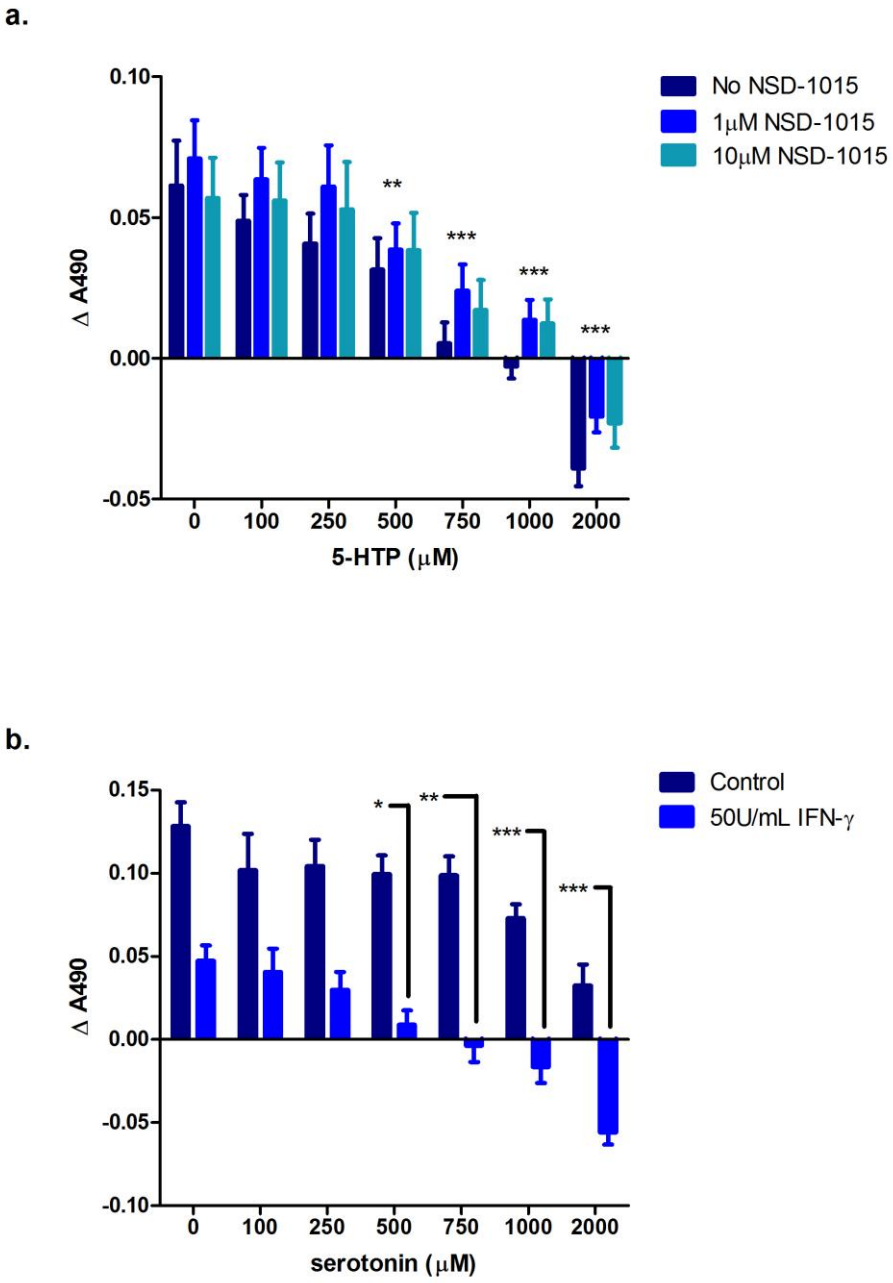


Figure 4.5 SH-SY5Y cell proliferation following 5-HTP, serotonin and NSD-1015 treatment with IFN-γ. a. The effect of NSD-1015 on 5-HTP induced changes in cell proliferation following treatment with 50U/mL IFN-γ measured by SRB assay b. The effect of serotonin treatment in the presence of 50U/mL IFN-γ on cell proliferation as measured by SRB assay. ΔA490: Change in absorbance at 490nm compared to cells prior to treatment. Results are mean ± SEM of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ difference in comparison to 0 by ANOVA followed by least significant difference test.

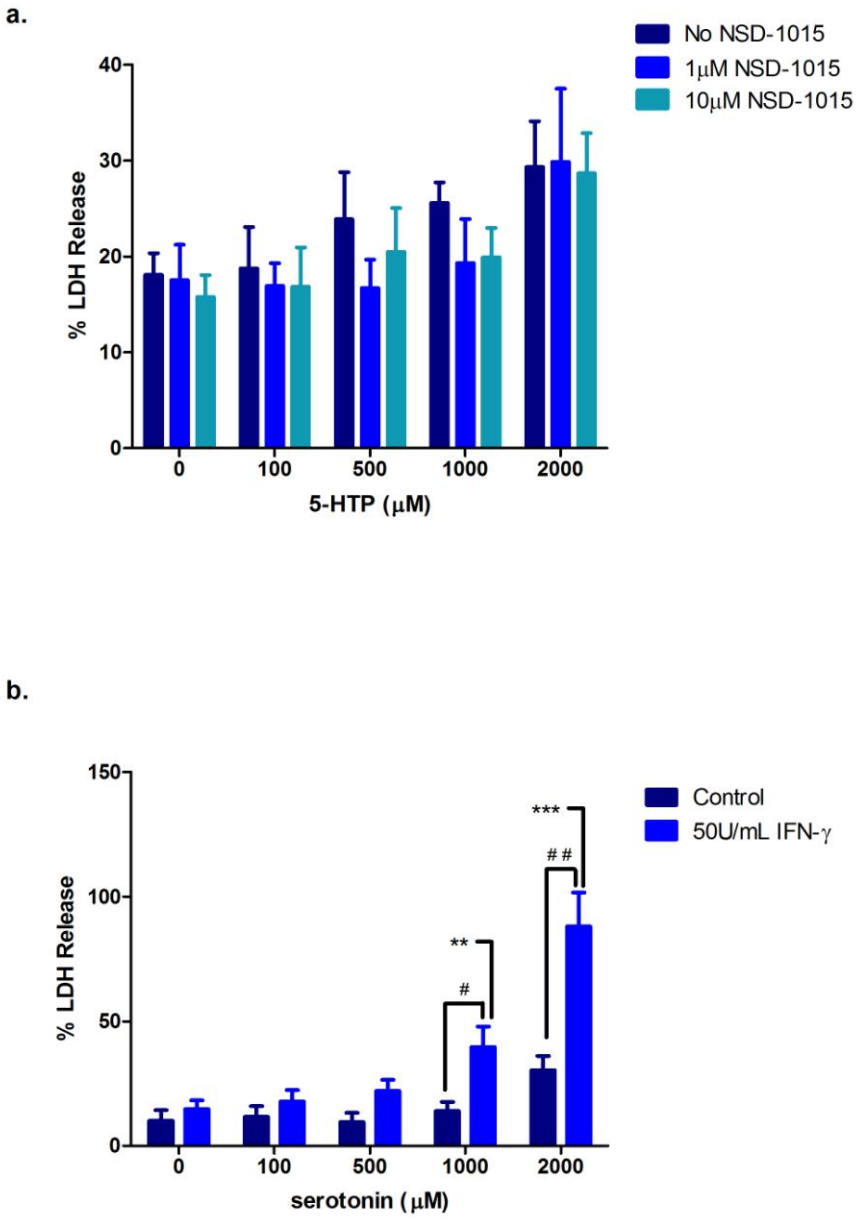


Figure 4.6 SH-SY5Y LDH release following 5-HTP, serotonin and NSD-1015 treatment with IFN-γ. a. The effect 5-HTP following treatment with NSD-1015 in the presence of 50U/mL IFN-γ on membrane integrity of SH-SY5Y cells measured by the LDH release assay. b. LDH release from SH-SY5Y cells in response to serotonin treatment in the presence of 50U/mL IFN-γ. % LDH release: LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis. Results are mean ± SEM of 5 independent experiments. ** $p < 0.01$, ***$p < 0.001$ difference in comparison to 0 by ANOVA followed by least significant difference test. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ determined by Student's t-test.

citrate synthase or as nmol/min/mg of protein. However the activity of each complex was not significantly different between cells treated with 5-HTP and IFN-γ and those treated with IFN-γ alone. Complex IV activity was also found to be increased following treatment with 5-HTP and IFN-γ in comparison to 5-HTP alone, however this was only significant when expressed as nmol/min/mg of protein ($p < 0.05$) and not significant as a ratio to citrate synthase activity. Combined treatment with 5-HTP, NSD-1015 and IFN-γ led to a significant increase in complex II/III activity in comparison to cells treated with 5-HTP and NSD-1015. However this effect was only significant when results were expressed as nmol/min/mg ($p < 0.05$), not when expressed as a ratio to citrate synthase. Furthermore complex activities were not significantly different between cells treated with 5-HTP, NSD-1015 and IFN-γ and those treated with NSD-1015 and IFN-γ. In comparison to serotonin alone, serotonin with IFN-γ treatment did lead to a significant increase in complex II/III activity when expressed as nmol/min/mg ($p < 0.05$), but not when expressed as a ratio to citrate synthase. However the activity of each of the complexes was not significantly different between serotonin and IFN-γ treated cells and cells treated with IFN-γ alone. Citrate synthase activity was not changed between any of the treatment conditions ($p = 0.999$; see table 4.2). Total protein levels were also not significantly different between any of the treatments ($p = 0.483$).

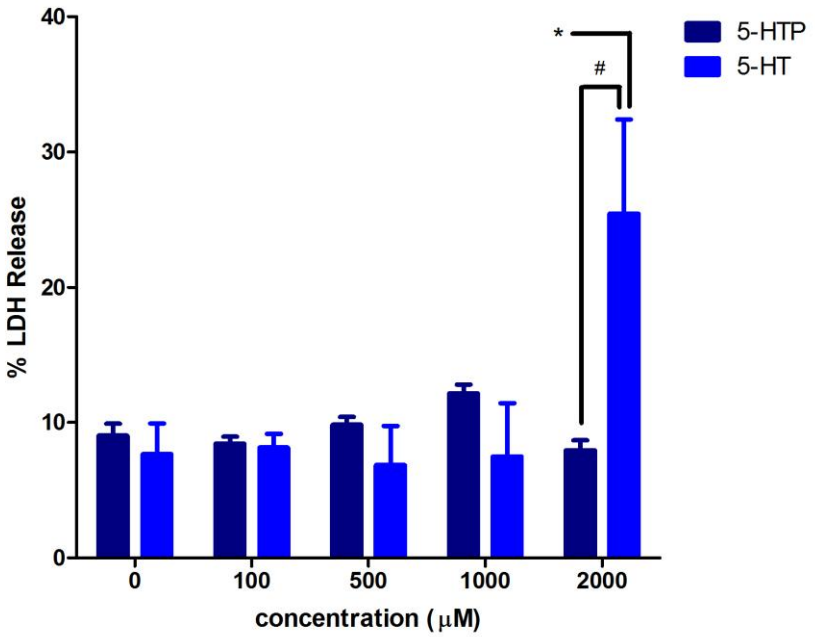


Figure 4.7 1321N1 LDH release following 5-HTP and serotonin treatment. The effect of 5-HTP and serotonin treatment on membrane integrity of 1321N1 cells measured by the LDH release assay. % LDH release: LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis. Results are mean ± SEM of 3 - 5 independent experiments. * $p < 0.05$ difference in comparison to 0 by ANOVA followed by least significant difference test. $^{\#}$ $p < 0.05$ determined by Student's t-test.

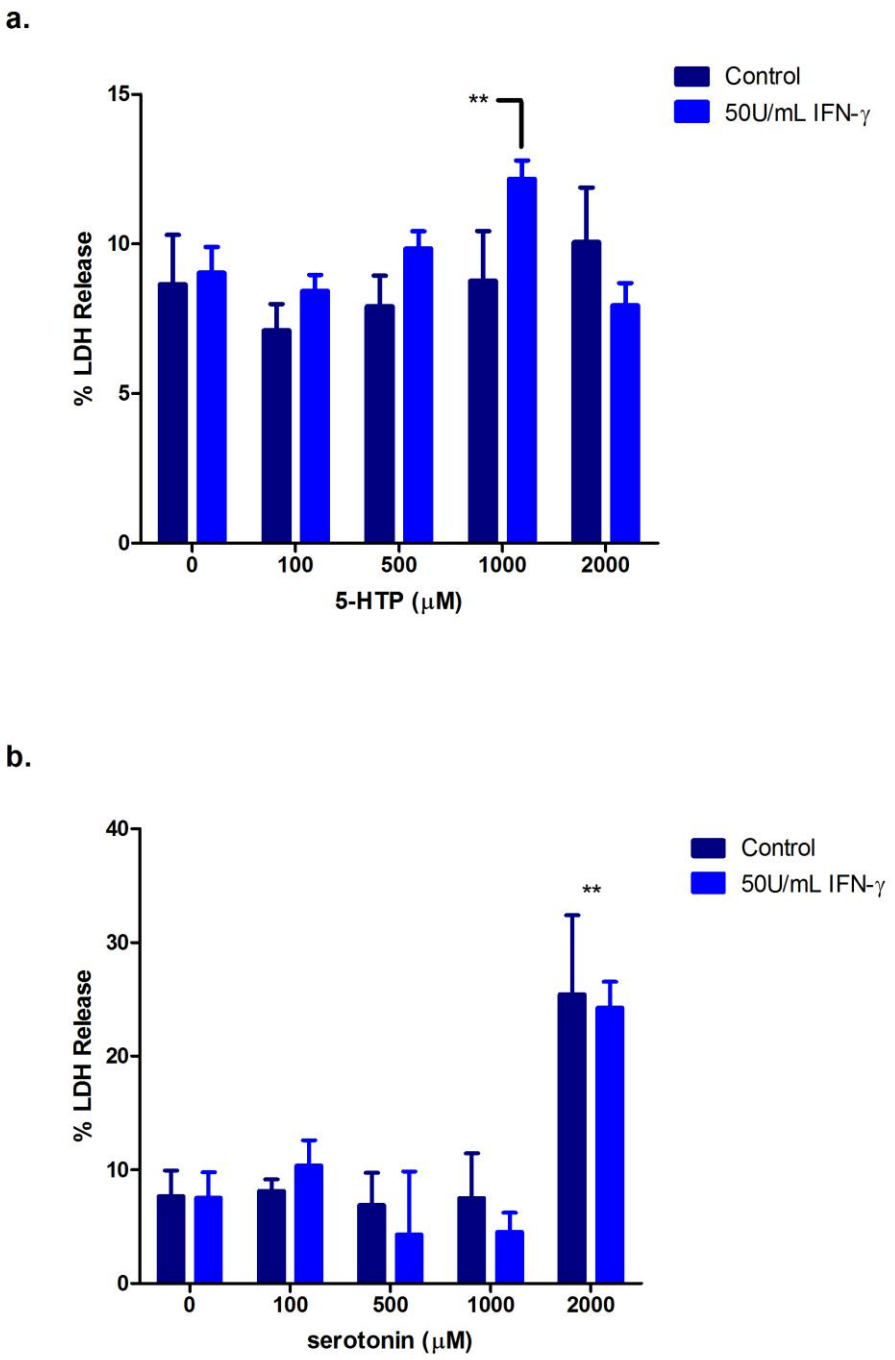


Figure 4.8 1321N1 LDH release following 5-HTP and serotonin treatment with IFN-γ. a. The effect of 5-HTP treatment in the presence or absence of 50U/mL IFN-γ on membrane integrity of 1321N1 cells measured by the LDH release assay. b. LDH release from 1321N1 cells in response to serotonin in the presence or absence of 50U/mL IFN-γ. % LDH release: LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis. Results are mean ± SEM of 3 - 5 independent experiments. ** $p < 0.01$ difference in comparison to 0 by ANOVA followed by least significant difference test.

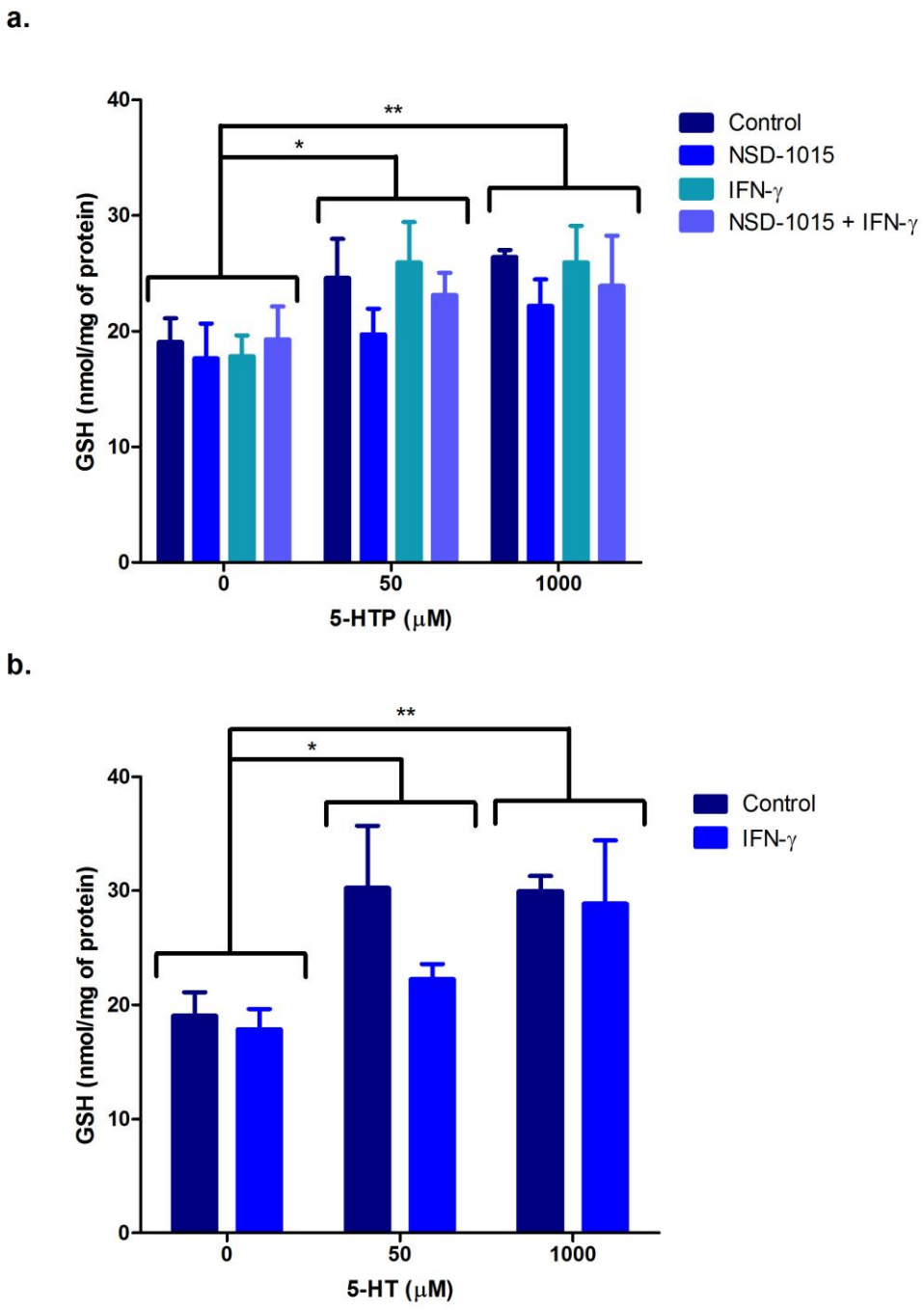


Figure 4.9 Intracellular GSH following 5-HTP or serotonin treatment in SH-SY5Y cells. a. Intracellular GSH in SH-SY5Y cells following treatment with 5-HTP in the presence or absence of 10μM NSD-1015 and/or 50U/mL IFN-γ. b. Intracellular GSH in SH-SY5Y cells following treatment with serotonin in the presence or absence of 50U/mL IFN-γ. Results are mean ± SEM of 3 independent experiments. *p <0.05, ** p <0.01 difference in comparison to 0 by ANOVA.

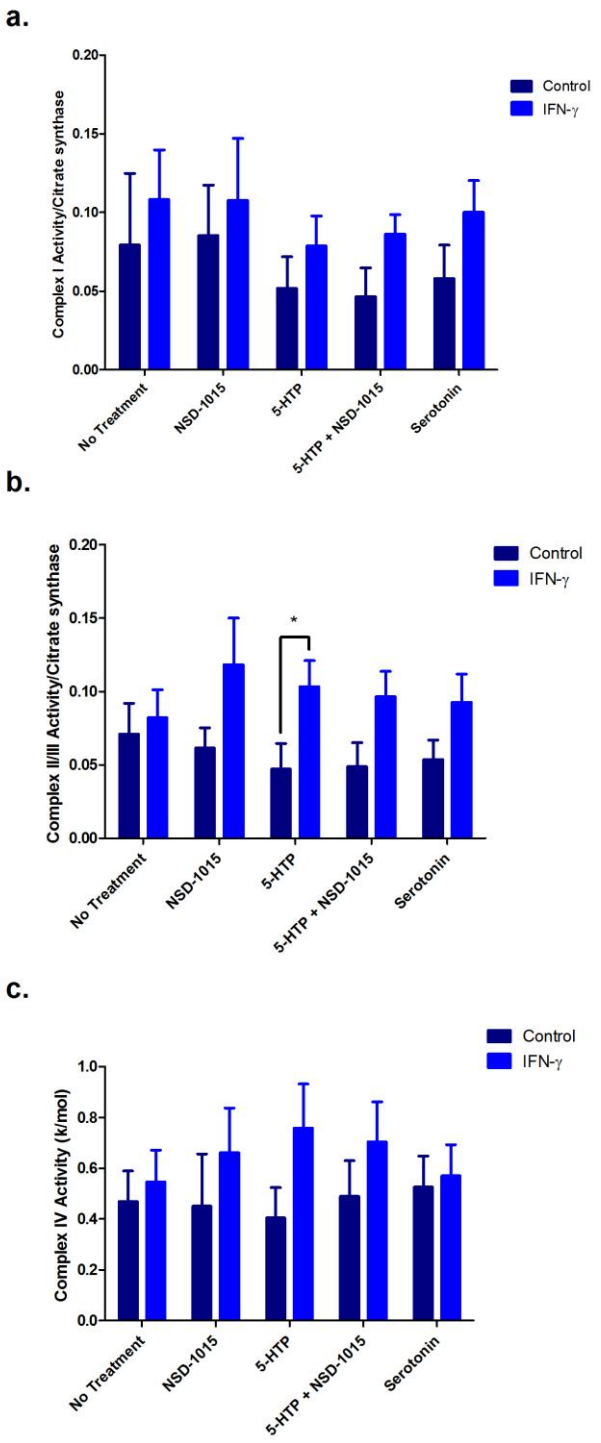


Figure 4.10 SH-SY5Y cell activity of mitochondrial respiratory chain after serotonin and 5-HTP treatment. a. complex I, b. complex II/III and c. complex IV expressed as a ratio to citrate synthase. Cells were treated with 50µM 5-HTP or serotonin as indicated for 48hr. 10µM NSD-1015 and/or 50U/mL IFN-γ was added 24hr before other treatments. Results are mean ± SEM from 3 - 4 independent experiments. * $p < 0.05$ determined by ANOVA followed by least significant difference test.

	Complex I		Complex II/III		Complex IV	Citrate synthase
	Activity	% inhibition	Activity	% inhibition		
Control	8.6 ± 3.5	54 ± 5	8.2 ± 1.0	98 ± 5	55.1 ± 6.4	146.1 ± 59.4
5-HTP	7.4 ± 1.9	60 ± 10	6.7 ± 1.3	104 ± 3	58.9 ± 9.0	162.7 ± 22.5
5-HTP + NSD-1015	4.9 ± 1.7	50 ± 27	6.2 ± 0.8	99 ± 6	64.1 ± 12.5	158.4 ± 46.2
5-HTP + IFN-γ	9.9 ± 2.1	63 ± 14	12.5 ± 1.0^{+}	103 ± 3	85.9 ± 7.9^{+}	136.9 ± 36.4
5-HTP + NSD-1015 + IFN-γ	9.9 ± 0.8	71 ± 6	10.8 ± 0.8$^{\$}$	100 ± 2	75.5 ± 5.5	126.3 ± 28.6
Serotonin	5.9 ± 1.3	48 ± 15	6.2 ± 0.7	100 ± 1	61.0 ± 8.5	134.6 ± 31.8
Serotonin + IFN-γ	11.0 ± 2.9	72 ± 7	9.8 ± 2.5$^{@}$	96 ± 6	56.2 ± 9.9	131.0 ± 53.2
NSD-1015	10.7 ± 1.8	73 ± 3	8.4 ± 0.3	99 ± 5	55.1 ± 14.6	159.0 ± 50.6
IFN-γ	14.0 ± 2.6	78 ± 0	11.0 ± 1.7	101 ± 1	73.8 ± 12.7	144.0 ± 25.3
NSD-1015 + IFN-γ	10.3 ± 2.2	55 ± 0	12.2 ±1.1	100 ± 1	68.3 ± 5.5	123.7 ± 39.1

Table 4.2 SH-SY5Y cell activity of mitochondrial respiratory chain after serotonin and 5-HTP treatment. Activity of complex I (nmol/min/mg of protein); complex II/III (nmol/min/mg of protein) and complex IV (k/min/mg of protein) as well as citrate synthase activity (nmol/min/mg). Complex I % inhibition following addition of rotenone and complex II/III % inhibition following addition of antimycin A. Cells were treated with 50µM 5-HTP or serotonin as indicated for 48hr. 10µM NSD-1015 and/or 50U/mL IFN-γ was added 24hr before other treatments. Results are mean ± SEM from 3 - 4 independent experiments. $^{+}$ $p < 0.01$ difference from 5-HTP alone, $^{\$}$ $p < 0.05$ difference from 5-HTP + NSD-1015, $^{@}$ $p < 0.05$ difference from serotonin alone all determined by ANOVA followed by least significant difference test.

4.6 Discussion

4.6.1 5-HTP and serotonin metabolism

Due to the lack of indoleaminergic cell lines the catecholaminergic SH-SY5Y cell line, which was found to express AADC mRNA, protein and activity was used to investigate the role of 5-HTP in AADC deficiency pathogenesis. The addition of 5-HTP to SH-SY5Y cells led to the production of serotonin and 5-HIAA demonstrating that serotonin could be produced and degraded in these cells. The degradation of serotonin to 5-HIAA is most likely via MAO-A that is known to be expressed in SH-SY5Y cells (Fitzgerald *et al*, 2007). Taken together these results suggest that indoleamine metabolism can be successfully reproduced in SH-SY5Y cells following the addition of 5-HTP. Furthermore following the extracellular addition of serotonin to SH-SY5Y cells intracellular serotonin was detected demonstrating that this cell line was capable of serotonin uptake. Additionally mRNA from the 5-HT_{2B} receptor has been isolated from SH-SY5Y cells, suggesting that this receptor type may be present at the cell membrane (Schmuck *et al*, 1994). Together these results suggested that SH-SY5Y cells could provide a useful model of some aspects of indoleamine physiology and could be used for initial testing of the hypotheses of this chapter.

NSD-1015 was used to inhibit AADC which, following 5-HTP treatment, led to reductions in the level of 5-HIAA and increases in 5-HTP, in a similar pattern to what is observed in the CSF of patients with AADC deficiency (Hyland and Clayton, 1990; Brun *et al*, 2010). Extracellular serotonin was also decreased following NSD-1015 treatment however intracellular serotonin was unaffected. This could suggest that intracellular serotonin was preserved in these cells at the expense of extracellular serotonin. Consequently this indicates that one possible adaptation to reduced serotonin biosynthesis in AADC deficiency could be to decrease serotonin release or increase re-uptake. Through measurement of quantal release of serotonin from presynaptic membranes it has been demonstrated that the amount of neurotransmitter contained within each small synaptic vesicle may be proportional to the size of the vesicle (Bruns *et al*, 2000). Potentially then serotonin deficiency could lead to less vesicles being filled and consequently serotonin release would be reduced. Alternatively serotonin deficiency could lead to an upregulation of SERT, increasing reuptake and recycling of serotonin, supporting this chronic blockade of serotonin re-uptake with cocaine leads to upregulation of SERT in humans (Mash *et al*, 2000).

IFN-γ was utilised throughout this study to activate IDO and consequently the kynurenine pathway. It has been shown previously that IFN-γ can activate IDO in neuronal cell lines including the parental cell line to SH-SY5Y cells, SK-N-SH (Guillemin *et al*, 2007). Activation of IDO would be expected to lead to an increase in the metabolism of 5-HTP and serotonin via the kynurenine pathway. However IFN-γ treatment did not lead to any decrease in 5-HTP or serotonin potentially indicating that they were not being degraded via this alternative metabolic route. This could suggest that either IDO was not active in this cell line or 5-HTP and serotonin were not degraded via the kynurenine pathway at the concentration used in these experiments. Treatment of SH-SY5Y cells with IFN-γ alone led to a reduction in cell growth, indicating that IFN-γ has an inhibitory effect on SH-SY5Y cell proliferation. This could relate to tryptophan depletion due to IDO activation, which has previously been shown to inhibit cell proliferation (Munn *et al*, 1999). Therefore this could indicate that IDO was active. IFN-γ treatment has also been demonstrated to lead to differentiation of some neuroblastoma cell lines, which in the mouse N103 cell line was dependent on $NO^{\bullet}$ production (Munoz-Fernandez *et al*, 1994; Ridge *et al*, 1996). IFN-γ is known to be a potent inducer of nitric oxide synthase (NOS) (Kilbourn and Bellomi, 1990; Petit *et al*, 1990) and both neuronal NOS (nNOS) and inducible NOS (iNOS) have previously been demonstrated to be expressed in SH-SY5Y cells (Russo *et al*, 2004; Guo *et al*, 2005). It is possible therefore that IFN-γ treatment increased $NO^{\bullet}$ production in SH-SY5Y cells during the current study. $NO^{\bullet}$ has been demonstrated to be an inhibitor of IDO (Thomas *et al*, 1994; Alberati-Giani *et al*, 1997) and therefore it is possible that while IDO expression was induced by IFN-γ treatment of SH-SY5Y cells, iNOS could also have been induced leading to inhibition of IDO via $NO^{\bullet}$.

The K_m of purified human IDO for 5-HTP is approximately 20-fold greater than that of tryptophan (Takikawa *et al*, 1988), indicating that 5-HTP would need to be in a great excess to compete with tryptophan as a substrate for IDO. The cell culture medium used with SH-SY5Y cells in the current study contains 44μM L-tryptophan (Invitrogen), almost equimolar with the concentration of 5-HTP (50μM). However if IDO was active it would seem likely, given its proposed physiological role in L-tryptophan depletion (Byrne *et al*, 1986; Beatty *et al*, 1994; Munn *et al*, 1999), that this initial concentration of L-tryptophan would undergo a relatively rapid degradation. Furthermore an L-tryptophan load in hens has been demonstrated to lead to the production of 5-hydroxykynurenine, demonstrating that in a physiological setting 5-

HTP can still be metabolised via IDO even under conditions of high L-tryptophan (Kido *et al*, 1967a Kido *et al*, 1967b). It is possible that at the 48hr time point, when indoleamines were measured in the current study, the levels of 5-HTP and serotonin could have been below the level required for IDO metabolism. Potentially at earlier time points, when 5-HTP and serotonin concentrations were likely to have been higher, there could have been some observable differences in indoleamine levels as a result of IFN-γ treatment. 5-hydroxykynurenine metabolites could not be directly measured as no synthetic 5-hydroxykynurenines were available for use as standards. For future work labelled [^{14}C-3]-5-HTP could be used to follow the metabolic fate of 5-HTP and identify if kynurenine metabolites do accumulate in an AADC deficient cell model (Zamboni and Sauer, 2009; Hiller *et al*, 2010).

4.6.2 5-HTP and serotonin toxicity

Greater than 500μM 5-HTP was found to be growth inhibitory to SH-SY5Y cells and LDH release was also slightly increased at higher concentrations of 5-HTP. Together these results indicate that 5-HTP may be mildly toxic to SH-SY5Y cells. Both the effect on cell growth and LDH release were unaffected by NSD-1015 demonstrating that these effects were not dependent on conversion to serotonin. However serotonin itself was demonstrated to be growth inhibitory and to increase LDH release to a greater extent than 5-HTP, however this was only observed at a concentration of 2000μM. This possible toxicity of 5-HTP and serotonin could relate to an increase in oxidative stress as both are capable of undergoing oxidation, however both 5-HTP and serotonin have been suggested to act as antioxidants (Ng *et al*, 2000; Herraiz and Galisteo, 2004; Munoz-Castaneda *et al*, 2005; Munoz-Castaneda *et al*, 2006). Alternatively it has been suggested that activation of serotonin 5-HT_2 receptors may lead to activation of apoptosis through increased intracellular Ca^{2+} (Schmidt *et al*, 1990; Azmitia, 2001). Consequently the increase in LDH release observed following serotonin treatment could relate to Ca^{2+} influx and excitotoxicity similar to that described for glutamate (reviewed by Fan and Raymond, 2007).

IFN-γ alone reduced cell growth and led to a small increase in cell lysis as measured by LDH release. This treatment has previously been demonstrated to inhibit SH-SY5Y cell growth (Martin *et al*, 1993; Ridge *et al*, 1996). SH-SY5Y cell populations contain two morphologically distinct but interconvertable cellular phenotypes neuroblast-like cells and epithelial-like cells (Biedler *et al*, 1978; Ciccarone *et al*, 1989). Ridge *et al* (1996)

demonstrated that IFN-γ treatment of SH-SY5Y cells induces differentiation of both phenotypes and also noted the presence of small-round cells in suspension in the media which the authors described as dead cells. In the present study combined treatment with >1000μM 5-HTP and IFN-γ led to a loss of cells, but with no concomitant increase in LDH release. This suggests that SH-SY5Y cells are detaching from the growth surface, but may not be dying as no change in membrane integrity was observed. This could indicate that the combined treatment of 5-HTP and IFN-γ causes differentiation of SH-SY5Y cells into a cell type that grows in suspension. Alternatively the cells may have been undergoing a cell death process but membrane integrity had not been disrupted at the time point when LDH release was measured. In support of this combined treatment of serotonin and IFN-γ led to both a decrease in cell growth and increased cell lysis, indicating that this treatment is toxic to SH-SY5Y cells. Furthermore NSD-1015 at 1μM but not 10μM decreased the effect of 5-HTP and IFN-γ on cell loss, suggesting that cell loss was partially dependent on conversion to serotonin and consequently may be related to cellular toxicity.

As measurement of indoleamines following IFN-γ treatment indicate that 5-HTP and serotonin may not have been degraded by the kynurenine pathway (see section 1.7.3) it seems unlikely that the toxicity of serotonin and 5-HTP following IFN-γ treatment could be related to the production of 5-hydroxykynurenine metabolites. However IFN-γ could have induced the production of L-tryptophan kynurenine metabolites and consequently there may potentially have been a synergistic effect between these metabolites and serotonin and 5-HTP. For example the L-tryptophan kynruenine metabolite quinolinic acid is a glutamate NMDA receptor agonist that causes excitotoxicity through calcium influx via NMDA receptors (Garthwaite and Garthwaite, 1986, Garthwaite and Garthwaite, 1987; Schurr *et al*, 1991). Additionally quinolinic acid induces oxidative stress through production of the $OH^{\bullet -}$ (Santamaria *et al*, 2001). Likewise another L-tryptophan kynurenine metabolite 3-hydroxykynurenine induces oxidative stress and cellular toxicity through the production of reactive oxygen species and also potentiates quinolinic acid induced toxicity (Eastman and Guilarte, 1989; Okuda *et al*, 1998; Guidetti and Schwarcz, 1999). Alternatively IFN-γ treatment could have led to IDO activation and consequently L-tryptophan depletion (Munn *et al*, 1999), leading to the observed inhibition of SH-SY5Y cell growth. In this state the cells may have been more vulnerable to high concentrations of serotonin or 5-HTP. An alternative explanation could possibly relate to increased $NO^{\bullet}$ production following

IFN-γ treatment (Bolanos *et al*, 1994; Bolanos *et al*, 1996; Minc-Golomb *et al*, 1996) which could potentially occur in SH-SY5Y cells as they are known to express iNOS (Russo *et al*, 2004; Guo *et al*, 2005). Increased levels of $NO^{\bullet}$ could increase the cellular oxidative burden and potentially make the cells more susceptible to damage induced by serotonin or 5-HTP treatment.

In contrast to SH-SY5Y cells LDH release from the human astrocytoma cell line 1321N1 was not increased following IFN-γ treatment. Furthermore although treatment with high concentrations of serotonin led to increases in LDH release from 1321N1 cells this effect was not changed by IFN-γ. This could indicate that neuronal-like cells are more susceptible than astrocyte-like cells to the mechanisms of combined IFN-γ and serotonin toxicity, although both may be equally susceptible to the effects of serotonin alone. 5-HTP treatment alone also had no effect on LDH release from 1321N1 cells. Combined treatment with IFN-γ and 1000μM 5-HTP led to a small increase in LDH release perhaps indicating some toxicity of this treatment which again may relate to the kynurenine pathway or $NO^{\bullet}$. The same treatment led to a greater increase in LDH release in SH-SY5Y cells, perhaps indicating increased neuronal susceptibility to this treatment. In AADC deficiency the level of 5-HTP in the CSF is generally in the nanomolar range (patient range: 51-264 nM; Brun *et al*, 2010). The concentration of 5-HTP has not been measured in close proximity to serotoninergic neurons though it is likely to be somewhat higher than in the CSF. This concentration may still not reach the level that was mildly toxic in the current experiments, however the neurons of patients with AADC deficiency are chronically exposed to 5-HTP, whereas in these experiments only acute effects were measured. Consequently the chronic effects of 5-HTP exposure require further investigation. Furthermore Guillemin *et al* (2005b) demonstrated that while neurons and astrocytes were able to express IDO, they were unable to produce quinolinic acid, a product of L-tryptophan kynurenine metabolism. In contrast microglial cells were able both to express IDO and produce quinolinic acid. This suggests that microglial cells rather than neurons or astrocytes, may be required for metabolism of 5-HTP via the kynurenine pathway. A co-culture system utilising both neuronal cells and microglial cells could therefore be an important future experiment to investigate the potential role of microglia in 5-HTP kynruenine metabolism and possible toxicity.

4.6.3 Glutathione and mitochondrial respiratory chain activity

GSH acts as a major cellular antioxidant acting as both a free radical scavenger as well as a substrate for glutathione peroxidise (reviewed by Martin and Teismann, 2009; Ballatori *et al*, 2009). 5-HTP and serotonin treatment led to small increases in the intracellular level of GSH in SH-SY5Y cells. This could be an indication of oxidative stress as GSH can increase in response to ROS (Gegg *et al*, 2003). γ-glutamylcysteine ligase can be upregulated by the activation of the transcription factor nuclear factor erythroid 2-related factor (Nrf2) that binds to promoter sequences termed antioxidant response elements (AREs; Rushmore *et al*, 1991; Venugopal *et al*, 1996; Itoh *et al*, 1997; Wild *et al*, 1999). Nrf2 activity is partly regulated by the cytosolic protein kelch-like ECH associating protein 1 (Keap1; Itoh *et al*, 1999; Zipper and Mulcahy, 2002; Itoh *et al*, 2003). Keap1 can act as a sensor for ROS and under oxidative stress this can lead to activation of Nrf2 (reviewed by Motohashi and Yamamoto, 2004). It is possible therefore in the current experiments that serotonin and 5-HTP led to an increase in ROS production that activated this pathway resulting in an upregulation of γ-glutamylcysteine ligase and the observed increase in GSH levels. However it has previously been demonstrated that serotonin is unable to activate Nrf2 at concentrations <100μM in astrocytes and meningeal cells (Shih *et al*, 2007). Conversely serotonin and 5-HIAA are able to inhibit lipid peroxidation and $OH^{\bullet -}$ formation *in vitro* (Ng *et al*, 2000). 5-HTP, serotonin and a range of other indolic compounds can also act as free radical scavengers in an *in vitro* system (Herraiz and Galisteo, 2004). This property of indoles is suggested to relate to the ability of the indolic nitrogen to accept a single electron. Depletion of serotonin led to a decrease in GSH levels in rats, which could be restored by subsequent administration of 5-HTP (Munoz-Castaneda *et al*, 2006). Additionally during serotonin depletion catalase and SOD activities were reduced and lipid peroxidation increased. Serotonin depletion has also been demonstrated to exacerbate lipid peroxidation, as well as catalase and SOD depletion in rats treated with the antibiotic gentamicin, which is known to induce oxidative stress (Munoz-Castaneda *et al*, 2005). It is possible therefore in the present study that serotonin and 5-HTP directly contributed to the antioxidant potential of SH-SY5Y cells thereby leading to the observed increases in GSH. Alternatively 5-HTP and 5-HIAA, but not serotonin, have been shown to inhibit glutathione S-transferase (Sawicki *et al*, 2001). Inhibition of this enzyme could reduce the consumption of GSH in conjugate formation potentially leading to an increase in intracellular GSH concentrations. Glutathione S-transferases

are involved in the detoxification of a range of endogenous and xenobiotic compounds by forming conjugates between GSH and the compound (reviewed by Hayes *et al*, 2005). It is possible therefore that whilst 5-HTP could increase GSH via inhibiting glutathione S-transferase, this would have a negative impact on cell survival and potentially redox status by reducing clearance of toxic compounds.

The mitochondrial respiratory chain is known to be susceptible to oxidative damage due to increased cellular ROS (Zhang *et al*, 1990; Benzi *et al*, 1991; Bolanos *et al*, 1994; Heales *et al*, 1995; Bolanos *et al*, 1996; Merad-Boudia *et al*, 1998; Merad-Saidoune *et al*, 1999; Chinta and Andersen, 2006). Mitochondria are also known to play a major role in cell survival, calcium signalling and apoptosis (Carafoli and Crompton *et al*, 1978; Miller, 1991; Neame *et al*, 1998; Nicholls and Budd, 2000; Pena *et al*, 2001). Consequently the activity of the respiratory chain enzyme complexes were investigated to determine whether 5-HTP or serotonin can induce changes in mitochondrial function. Combined treatment of SH-SY5Y cells with IFN-γ and 5-HTP or serotonin appeared to lead to an increase in the activity of complex II/III compared to cells treated with serotonin or 5-HTP alone. For serotonin or 5-HTP with NSD-1015 this effect was only significant when expressed as a proportion of cellular protein and not significant when expressed as a ratio to citrate synthase activity. Citrate synthase activity is commonly used as a mitochondrial marker and as a measure of mitochondrial enrichment (Winder and Holloszy, 1977; Fatania and Dalziel, 1980; Hargreaves *et al*, 2007). Citrate synthase activity was unaffected by any of the treatments used in the current experiments. This suggests that the effect on complex II/III does not relate to changes in the amount of mitochondria. Likewise total protein was unaffected by any of the treatments which indicates that the observed effects on complex II/III are not an artefact from a change in cell number. Furthermore the % antimycin A inhibition during the assay of complex II/III was not altered between treatments, indicating that non-specific reduction of cytochrome c was not responsible for the observed differences in complex II/III activity. The lack of significance in results expressed as a ratio to citrate synthase could be explained by a higher level of variation in these results compared to results expressed as a proportion of total protein. Consequently the results could indicate that the combination of IFN-γ and 5-HTP or serotonin does lead to an increase in complex II/III activity. Furthermore the effect of 5-HTP was not abolished by co-incubation with NSD-1015 suggesting that conversion of 5-HTP to serotonin was not required for this effect.

One possible explanation for this synergistic effect may relate to an increase in mitochondrial antioxidant status. 5-HTP or serotonin treatment led to a small increase in GSH, which may relate in part to the potential ability of indoles to act as free radical scavengers (Ng *et al*, 2000; Herraiz and Galisteo, 2004; Munoz-Castaneda *et al*, 2006). IFN-γ treatment has previously been demonstrated to increase MnSOD, which is responsible for the conversion of $O_2^{\bullet-}$ to H_2O_2 within mitochondria (Okado-Matsumoto and Fridovich, 2001). Potentially combined treatment with 5-HTP or serotonin and IFN-γ would increase both GSH and MnSOD, perhaps leading to an increased efficiency in $O_2^{\bullet-}$ detoxification via SOD and subsequently H_2O_2 reduction via glutathione peroxidise. Together this could lead to a lower level of ROS mediated damage to complexes II and III leading to enhanced activity. Furthermore the L-tryptophan kynurenine metabolite kynurenic acid has been demonstrated to protect against toxicity of the MPP^+ complex I inhibitor by reducing the level of the pro-apoptotic Bax protein (Lee *et al*, 2008c). Part of this protection was elicited by preventing depolarisation of the mitochondrial membrane.

The increase in complex II/III activity in response to 5-HTP or serotonin and IFN-γ could indicate that this treatment affords some protection to neuronal-like cells leading to stimulation of oxidative phosphorylation. However IFN-γ treatment has previously been demonstrated to lead to differentiation of SH-SY5Y cells (Ridge *et al*, 1996) and possibly the combined effect of indoles and IFN-γ altered the phenotype of these cells resulting in an increase in oxidative phosphorylation. IFN-γ can also activate iNOS leading to $NO^{\bullet}$ production (Bolanos *et al*, 1994; Bolanos *et al*, 1996; Minc-Golomb *et al*, 1996). $NO^{\bullet}$ has been demonstrated to be an inhibitor of complex IV activity (Bolanos *et al*, 1994; Heales *et al*, 1994; Brown *et al*, 1995). Both microglial cells and astrocytes have been demonstrated to produce $NO^{\bullet}$ in response to IFN-γ stimulation (Chao *et al*, 1992; Demerle-Pallardy *et al*, 1993; Corradin *et al*, 1993; Colasanti *et al*, 1993). Furthermore the increase in astrocyte produced $NO^{\bullet}$ has been shown to have a negative impact on neuronal respiration (Bolanos *et al*, 1996; Stewart *et al*, 2000). Consequently further work would need to be conducted to ascertain whether the combined effect of IFN-γ and 5-HTP or serotonin would increase neuronal complex II/III function in the presence of glial cells.

4.7 Conclusions

AADC deficiency results in a deficit of serotonin and an accumulation of the precursor 5-HTP in patient CNS. The evidence presented in this chapter indicates that 5-HTP could be mildly toxic to neuronal-like cells. The concentration range at which toxicity was observed was above that seen in patients, however it is possible that chronic exposure to 5-HTP could cause neuronal damage at lower concentrations. 5-HTP and serotonin were both found to increase intracellular GSH in neuronal-like cells. This could indicate that indoles contribute to enhancing the antioxidant status of neurons, although 5-HTP has previously been shown to inhibit glutathione S-transferase, which could have a negative effect on detoxification of damaging compounds. IFN-γ a known activator of the kynurenine pathway, was found to act synergistically with both 5-HTP and serotonin, leading to some neuronal toxicity at higher concentrations of indoles. However it is possible that in these investigations the kynurenine pathway was not activated and therefore the role of kynurenine metabolism in AADC deficiency requires further investigation. At sub-toxic concentrations IFN-γ in combination with 5-HTP or serotonin appeared to increase the activity of complex II/III of the respiratory chain, this could represent a beneficial effect of indoles and IFN-γ in enhancing antioxidant protection, however this effect could also relate to differentiation of neuronal-like cells.

Chapter 5

The effect of L-dopa and dopamine on cell viability, glutathione homeostasis and mitochondrial function

5.1 Introduction

L-dopa has been demonstrated to accumulate in the CSF of patients with AADC deficiency (Brun et al, 2010). Potentially the accumulation of this substrate may have damaging effects for patients. L-dopa and dopamine have been shown to be toxic to isolated primary neuronal cultures and to neuronal cell lines including the SH-SY5Y neuroblastoma cell line (Mena *et al*, 1993; Basma *et al*, 1995; Lai and Yu, 1997; Nakao *et al*, 1997; Offen *et al*, 2001; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003). This toxicity is primarily considered to be due to auto-oxidation of L-dopa and dopamine in cell culture medium inducing oxidative stress via ROS production. In these studies the inhibition of AADC with benserazide or carbidopa did not prevent the toxicity of L-dopa indicating that it's conversion to dopamine was not required for toxicity (Basma *et al*, 1995; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003). Much of the toxic effect of L-dopa and dopamine could be prevented by the extracellular addition of catalase, which converts H_2O_2 to $2H_2O$, demonstrating that extracellular generation of H_2O_2 was an important mechanism for toxicity (Lai and Yu, 1997). Additionally 3-OMD, a major L-dopa metabolite that accumulates to a greater extent than L-dopa in the CSF of AADC deficient patients, that does not auto-oxidise, has been shown not to be toxic in cell culture (Offen *et al*, 2001; Haque *et al*, 2003; Hyland *et al*, 1992; Maller *et al*, 1997; Brun et al, 2010). It has also been suggested that the *in vitro* toxicity of L-dopa may be an artefact of cell culture (Clement *et al*, 2002). Furthermore *in vivo* studies indicate that chronic L-dopa administration does not cause damage to the dopaminergic nigrostriatal pathway (Hefti *et al*, 1981; Perry *et al*, 1984; Quinn *et al*, 1986; Zeng *et al*, 2001; Mytilineou *et al*, 2003).

The disparity between *in vivo* and *in vitro* toxicity of L-dopa and dopamine may in part relate to catalysis of auto-oxidation by some components of cell culture medium (Clement *et al*, 2002). However it has also been demonstrated that glial-conditioned medium protects primary neuronal cultures from L-dopa induced toxicity (Mena *et al*, 1996; Mena *et al*, 1997b). In addition L-dopa was not toxic to neurons in a neuronal-astrocyte co-culture system (Mena *et al*, 1997a). This evidence indicates that glial cells, and particularly astrocytes may play a role *in vivo* in preventing the L-dopa or dopamine induced toxicity seen in isolated neuronal culture. L-dopa may also have some neuroprotective effects. The intracellular levels of GSH have been found to be increased in response to L-dopa treatment in both isolated neuronal culture and in

neuronal/astrocyte co-culture (Mytilineou *et al*, 1993; Mena *et al*, 1997a). Dopamine has also been demonstrated to increase GSH levels in rat pheochromocytoma cells (Jia *et al*, 2008a). However Han *et al* (1996) found that inhibition of AADC did not prevent the L-dopa induced rise in GSH in mouse neuroblastoma and pig kidney epithelial cells, indicating that conversion to dopamine was not required for the effect. Furthermore pre-treatment with L-dopa or dopamine protected cells against cell death following subsequent treatment with inducers of oxidative stress (Han *et al*, 1996; Jia *et al*, 2008a). Conversely dopamine has been demonstrated to negatively affect GSH release from primary astrocytes, which may relate to dopamine auto-oxidation that in turn may have led to oxidation of GSH (Hirrlinger *et al*, 2002). The effects of L-dopa upon GSH release from astrocytes were not investigated. GSH release is considered to be the first stage in trafficking of GSH from astrocytes to neurons consequently alterations in GSH release can lead to decreased GSH synthesis within neurons (see section 1.7.6, Dringen *et al*, 1999; Stewart *et al*, 2002)

Although L-dopa administration was not found to be toxic in wildtype animals recent studies indicate that alterations in the metabolism and/or storage of catecholamines may lead to neurodegeneration. Mice expressing low levels of VMAT2 displayed reduced storage and increased turnover of striatal dopamine and this was accompanied by degeneration of dopaminergic striatal neurons at 18 and 24 months of age (Caudle *et al*, 2007). Furthermore expression of DAT in striatal neurons unable to store or metabolise dopamine also induced neurodegeneration in mice which was exacerbated by L-dopa administration (Chen *et al*, 2008a). Conversely viral gene transfer of AADC into cultured striatal neurons, which were able to store dopamine, protected these cells from L-dopa toxicity (Doroudchi *et al*, 2005). In this study the expression of AADC induced an increase in VMAT2 expression that potentially enhanced the ability of the neurons to store dopamine. These studies imply that alterations in dopamine homeostasis and in particular increased cytosolic dopamine or reduced dopamine storage can lead to neurodegeneration. In AADC deficiency conversion of L-dopa to dopamine is severely reduced. L-dopa accumulates in the CSF of AADC deficient patients and potentially therefore L-dopa may also be increased in the cytosol of dopaminergic neurons. The consequences of increased cytosolic L-dopa are unknown; however it could be similarly toxic to increased cytosolic dopamine. Furthermore dopamine has been demonstrated *in vitro* and in SH-SY5Y cells to reduce mitochondrial O_2 consumption and lead to mitochondrial membrane depolarisation (Cohen *et al*, 1997; Berman and Hastings,

1999; Gluck *et al*, 2002; Gluck and Zeevalk, 2004; Brenner-Lavie *et al*, 2008; Brenner-Lavie *et al*, 2009). These effects were related in part to ROS generation as well as effects of dopamine-quinone. It is possible that L-dopa accumulation could lead to similar changes in respiration which could impact upon the pathogenesis of AADC deficiency.

5.2 Aims

1) To determine the toxicity of L-dopa, 3-OMD and dopamine in a human neuronal cell line.
2) To investigate the effect of increased levels of L-dopa and dopamine on intracellular GSH and GSH release in human neuronal and astrocyte cell lines
3) To investigate the effect of L-dopa and dopamine on the mitochondrial respiratory chain

5.3 Methods

5.3.1 Treatment solutions

20mM NSD-1015, 10mM L-dopa, 10mM dopamine, 10mM 3-OMD, 10mM noradrenaline, 15KU/mL SOD, 3KU/mL catalase, 300µM tranylcypromine, 300µM Ro-41-0960, 300µM SCH-23390 and 300µM sulpiride were made up as stock solutions in HPLC grade H_2O, 0.2µM sterile filtered and stored at -20˚C.

5.3.2 Cell culture

Routine cell culture was performed as described in section 2.2.6 for SH-SY5Y cells and in section 2.2.7 for 1321N1 cells

5.3.3 Measurement of catecholamines

Cells and medium were prepared as described in section 2.2.8.1 and catecholamines measured as described in section 2.2.1

5.3.4 Measurement of GSH

Cells and minimal medium were prepared as described in section 2.2.8.2 and section 2.2.8.3 and GSH measured as described in section 2.2.4

5.3.5 SRB cell proliferation assay

The SRB cell proliferation assay was performed as described in section 2.3

5.3.6 LDH Release assay

The LDH release assay was performed as described in section 2.4

5.3.7 Mitochondrial respiratory chain complex assays and citrate synthase

The activity of complex I, II/III, IV and citrate synthase were determined by the methods described in section 2.7

5.4 Experimental Protocol

5.4.1 Catecholamine measurement

SH-SY5Y cells were seeded in 12.5mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 56.7cm^2 cell culture dishes. 6.25µL 20mM NSD-1015 (final conc. 10µM), 12.5µL 10mM tranylcypromine (final conc. 10µM) or 12.5µL 10mM Ro-41-0960 (final conc. 10µM) were added to appropriate dishes and 12.5µL sterile HPLC grade H_2O was added to untreated dishes. The concentration of NSD-1015 used was chosen based on investigations of indoleamines (see section 4.5.2). The concentration of tranylcypromine and Ro-41-0960 were chosen based on previous investigations (Blessing *et al*, 2003; Fitzgerald *et al*, 2007). Cells were incubated for 24hr at +37˚C in 5.0% CO_2 to allow cells to adhere. 62.5µL 10mM L-dopa (final conc. 50µM) or 10mM dopamine (final conc. 50µM) were added to appropriate dishes and cells incubated for 48hr at +37˚C in 5.0% CO_2. These concentrations of L-dopa and dopamine were chosen to minimise cellular toxicity whilst also maximising the availability of substrate for metabolism over the incubation period (see section 5.5.2). Cells and cell culture medium were then processed as indicated for catecholamine measurement (see section 5.3.3).

5.4.2 Toxicity testing

For SRB and LDH release assays SH-SY5Y cells were seeded in 96-well plates at a density of 1.80 x 10^4 cells/well in 180µL/well DMEM/F-12 + 100mL/L FBS. 10µL NSD-1015 (200µM: final conc. 10µM or 20µM: final conc. 1µM) was added to treated wells, 10µL sterile HPLC grade H_2O was added to untreated wells. Plates were incubated for 24hr to allow cells to adhere at +37˚C in 5.0% CO_2. 10µL L-dopa,

dopamine or 3-OMD (concentration range: 10mM to 10µM: final conc. 500µM to 0.5µM) were added to appropriate treated wells. 10µL sterile HPLC grade H_2O was added to control wells. Cells were then incubated for 48hr at +37˚C in 5.0% CO_2. Cells were then processed as indicated for the appropriate assay (see section 5.3.5 for the SRB assay and section 5.3.6 for LDH release assay).

For LDH release into minimal medium SH-SY5Y cells or 1321N1 cells were seeded at a density of 1.80 x 10^4 cells/well in 180µL DMEM/F-12 +100mL/L FBS or DMEM + 100mL/L FBS respectively. 10µL 200µM NSD-1015 (final conc. 10µM) was added to appropriate wells, 10µL sterile HPLC grade H_2O was added to all other wells. Plates were incubated at +37˚C in 5.0% CO_2 for 24hr to allow cells to adhere. 10µL 1mM L-dopa (final conc. 50µM) or dopamine (1mM: final conc. 50µM or 0.2mM: final conc. 10µM) were added to treated wells. 10µL sterile HPLC grade H_2O was added to control wells. Plates were incubated for the indicated length of time at +37˚C in 5.0% CO_2. Cell culture medium was removed, cells were washed once with 200µL/well HBSS and subsequently incubated at +37˚C in 5.0% CO_2 for 4hr. The LDH release assay was then performed as indicated (see section 5.3.6).

5.4.3 GSH measurement

SH-SY5Y cells were seeded in 3mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 6-well cell culture dishes, the surface area of each well was 9.6cm^2. 1321N1 cells were seeded at a density of 6.25 x 10^4 cells/cm^2 in 3mL DMEM + 100mL/L FBS in 6-well cell culture dishes. 150µL NSD-1015 (1mM: final conc. 50µM or 0.2mM: final conc. 10µM), 100µL 15KU/mL SOD (final. 500U/mL), 100µL 3KU/mL catalase (final. 100U/mL), 100µL 300µM tranylcypromine (final conc. 10µM) or 100µL 300µM Ro-41-0960 (final conc. 10µM) were added to appropriate treated wells. The concentration of SOD, catalase, tranylcypromine and Ro-41-0960 used were based on previous studies (Lai and Yu et al, 1997; Stewart *et al*, 2002; Blessing *et al*, 2003; Fitzgerald *et al*, 2007). 150µL sterile HPLC grade H_2O was added to untreated wells. Plates were incubated at +37˚C in 5.0% CO_2 for 24hr to allow cells to adhere. Where indicated 100µL 300µM SCH-23390 or sulpiride (final conc. 10µM) were added to appropriate wells and incubated for 1hr. The concentrations of SCH-23390 and sulpiride were chosen based on previous investigations (Bryson *et al*, 1992; Hall *et al*, 1993; Koshimura *et al*, 2000). 15µL L-dopa or dopamine (10mM: final conc. 50µM; 2mM: final conc. 10µM or 0.2mM: final conc. 1µM) were added to appropriate treated

wells. 15µL sterile HPLC grade H_2O was added to untreated wells. Plates were then incubated at +37˚C in 5.0% CO_2 for the indicated length of time. For determination of intracellular GSH cells were processed as indicated (see section 5.3.4). For determination of GSH release cell culture medium was removed, cells were washed once with 2mL/well HBSS and subsequently incubated for 4hr in 1mL/well minimal medium at +37˚C in 5.0% CO_2. Minimal medium was then removed and processed as indicated (see section 5.3.4).

5.4.4 Mitochondrial respiratory chain

SH-SY5Y cells were seeded in 30mL DMEM/F-12 + 100mL/L FBS at a density of 5.00 x 10^4 cells/cm^2 in 145cm^2 cell culture dishes. 15µL 20mM NSD-1015 (final conc. 10µM) was added to treated dishes and 15µL sterile HPLC grade H_2O was added to untreated dishes. Cells were incubated for 24hr at +37˚C in 5.0% CO_2 to allow cells to adhere. 150µL L-dopa (10mM: final conc. 50µM) or dopamine (10mM: final conc. 50µM) were added to appropriate dishes, 150µL sterile HPLC grade H_2O was added to control dishes. Cells were incubated for 48hr at +37˚C in 5.0% CO_2. Cells were then processed as indicated for respiratory chain complex assays and citrate synthase (see section 5.3.7).

5.5 Results

5.5.1 Effect of NSD-1015 on catecholamine metabolism in human neuroblastoma cells

SH-SY5Y cells were treated with 10µM NSD-1015 for 24hr at which point 50µM L-dopa was added and incubated for 24hr and 48hr. After these incubations the levels of L-dopa, dopamine and metabolites were measured in cell homogenates and in the cell culture medium. Consistent with inhibition of AADC the intra- and extracellular concentrations of L-dopa and 3-OMD were significantly increased, whilst the concentrations of dopamine, DOPAC and HVA were significantly decreased at both 24hr and 48hr in comparison to cells treated with L-dopa alone (see table 5.1). When NSD-1015 treated cells were incubated with 50µM dopamine in place of L-dopa there were significant reductions in the extracellular levels of DOPAC and HVA at both 24hr and 48hr, whilst extracellular dopamine was increased at 24hr in comparison to cells treated with dopamine only (see table 5.1). Similarly the intracellular level of DOPAC and HVA were significantly decreased at 24hr ($p < 0.001$, $p < 0.01$ respectively) and

HVA was also decreased at 48hr ($p < 0.05$). This could be indicative of reductions in MAO activity. Furthermore the extracellular concentration of 3-OMD was also significantly reduced at 48hr ($p < 0.01$) indicating that COMT may also be affected.

5.5.2 Effect of L-dopa and dopamine on neuroblastoma cell survival

The SRB assay was used to determine the effect on cell growth of a 48hr treatment of SH-SY5Y neuroblastoma cells with varying concentrations of L-dopa and dopamine. At a concentration of 50µM both L-dopa and dopamine were found to reduce cell growth by approximately 29% compared to untreated control ($p < 0.001$, $p < 0.05$ respectively; see figure 5.1a). L-dopa and dopamine treatment at concentrations >250µM led to a loss of cells relative to the amount of cells present before treatment. The overall effect of L-dopa and dopamine upon SH-SY5Y cells was found to be significantly different ($p < 0.001$). From individual comparisons this difference was significant only at lower concentrations from 0.5µM to 25µM, with no difference observed at ≥50µM (see figure 5.1a). At these concentrations L-dopa had less effect on cell proliferation in comparison to dopamine. 3-OMD had no effect on neuroblastoma cell proliferation at concentrations up to 500µM ($p = 0.989$). The effect of L-dopa on cell proliferation was also investigated in cells that had been treated with NSD-1015. NSD-1015 alone did not influence SH-SY5Y cell proliferation at either 1µM or 10µM ($p = 0.760$, $p = 0.263$ respectively). Overall 10µM NSD-1015 had a significant effect on the response of SH-SY5Y cells to L-dopa in comparison to cells treated with L-dopa alone ($p < 0.05$), however when each L-dopa concentration was compared individually 10µM NSD-1015 had no significant effect at any L-dopa concentration tested in comparison to cells treated with L-dopa alone. Treatment with 1µM NSD-1015 did not alter the effect of L-dopa treatment on SH-SY5Y cell proliferation ($p = 0.705$; see figure 5.1b).

In addition to cell proliferation the effect of L-dopa and dopamine on membrane integrity was investigated using the LDH release assay. Treatment of SH-SY5Y cells with 250µM L-dopa or dopamine for 48hr led to significant increases in LDH release to 83 ± 7% and 90 ± 10% of control respectively ($p < 0.001$ for both, see figure 5.2a). At concentrations of 100µM or below, L-dopa treatment had no effect on LDH release. Conversely dopamine treatment produced increases in LDH release at both 50µM and 100µM compared to control ($p < 0.05$, $p < 0.01$ respectively) and in comparison to L-dopa treatment ($p < 0.05$, $p < 0.001$ respectively). 3-OMD did not affect LDH release from SH-SY5Y cells up to a concentration of 250µM ($p = 0.903$; see figure 5.2a). The

Treatment	Incubation time (hr)	L-dopa	3-OMD	dopamine	DOPAC	HVA
INTRACELLULAR						
L-dopa control	24	27 ± 8	0 ± 0	919 ± 126	1020 ± 76	145 ± 33
	48	21 ± 6	2 ± 2	289 ± 46	255 ± 55	171 ± 31
L-dopa + NSD-1015	24	139 ± 19$^{###}$	31 ± 7$^{##}$	109 ± 12$^{###}$	9 ± 2$^{###}$	7 ± 2$^{##}$
	48	105 ± 13$^{###}$	30 ± 7$^{##}$	107 ± 14$^{##}$	11 ± 3$^{##}$	13 ± 5$^{###}$
dopamine control	24	33 ±9	0 ± 0	402 ± 39	850 ± 89	165 ± 18
	48	28 ± 7	2 ± 2	223 ± 51	103 ± 33	103 ± 22
dopamine + NSD-1015	24	38 ± 10	1 ± 1	518 ± 51	146 ± 18$^{###}$	75 ± 11$^{##}$
	48	28 ± 10	1 ± 1	223 ± 29	32 ± 6	46 ± 10$^{#}$
EXTRACELLULAR						
L-dopa control	24	14768 ± 1072	0 ± 0	3854 ± 425	4160 ± 474	1419 ± 198
	48	4689 ± 602	26 ± 26	1963 ± 200	3761 ± 311	4436 ± 542
L-dopa + NSD-1015	24	23351 ± 713$^{###}$	416 ± 115$^{##}$	145 ± 99$^{###}$	0 ± 0$^{###}$	0 ± 0$^{###}$
	48	16223 ± 1080$^{###}$	1266 ± 158$^{###}$	35 ± 35$^{###}$	180 ± 128$^{###}$	263 ± 105$^{###}$
dopamine control	24	960 ± 23	181 ± 57	19663 ± 1186	7100 ± 554	2120 ± 200
	48	945 ± 22	287 ± 35	2458 ± 380	4023 ± 428	4458 ± 538
dopamine + NSD-1015	24	958 ± 22	104 ± 44	28304 ± 739$^{###}$	1211 ± 142$^{###}$	447 ± 79$^{###}$
	48	947 ± 28	73 ± 44$^{##}$	1447 ± 389	452 ± 77$^{###}$	1350 ± 257$^{###}$

Table 5.1 Catecholamine levels following treatment with L-dopa or dopamine and NSD-1015. Cells treated with 10µM NSD-1015 for 24hr before the addition of 50µM L-dopa or dopamine. Intracellular values expressed as pmol/mg of protein and extracellular values expressed as nM. All values are mean ± SEM of 5 independent experiments. $^{#}$ $p < 0.05$, $^{##}$ $p < 0.01$, $^{###}$ $p < 0.001$ determined by Student's t-test comparing NSD-1015 treatment to control.

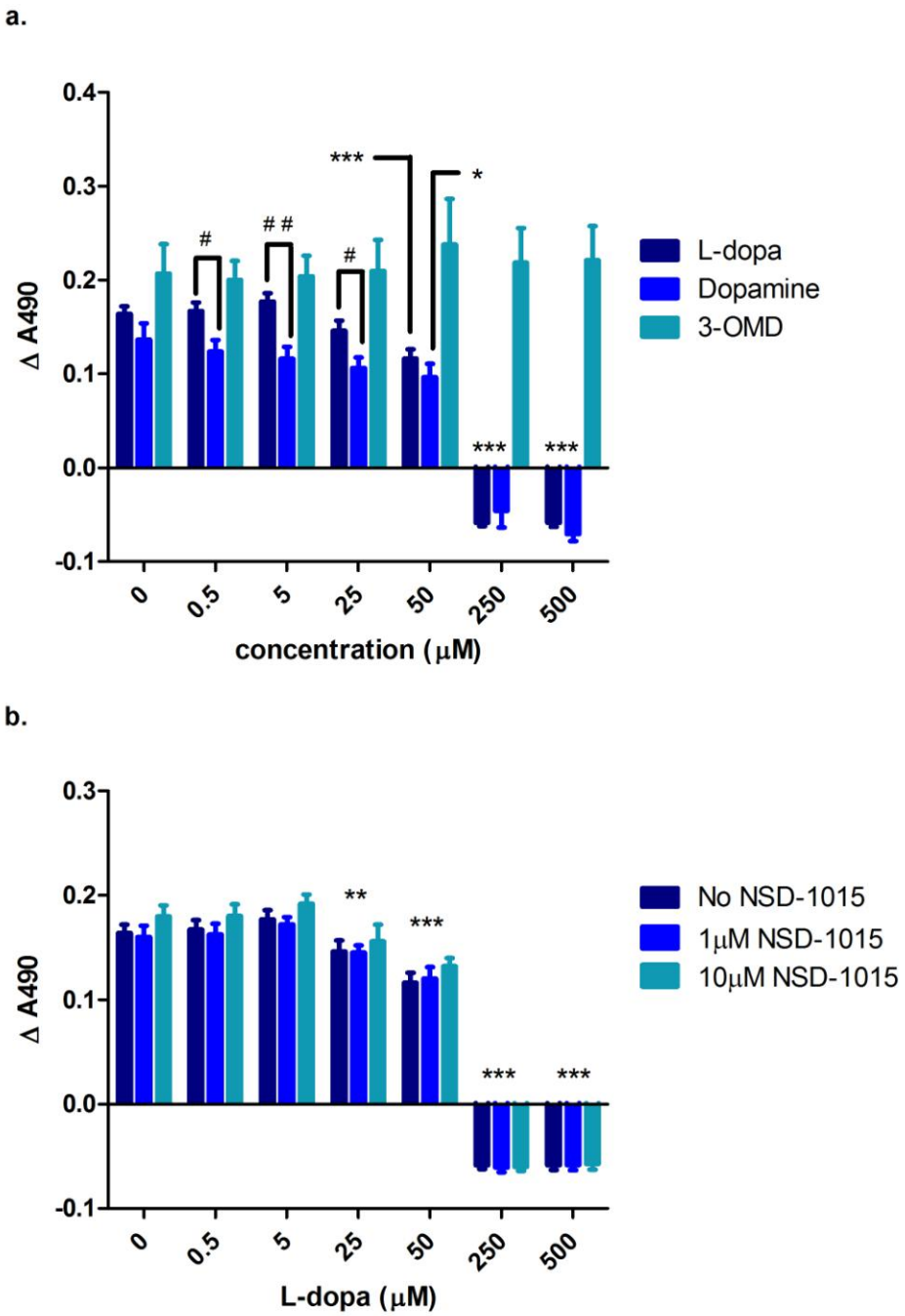


Figure 5.1 SH-SY5Y cell proliferation following L-dopa, dopamine, 3-OMD and NSD-1015 treatment. a. The effect of L-dopa, dopamine and 3-OMD treatment on cell proliferation as measured by SRB assay. b. The effect of NSD-1015 on L-dopa induced changes in cell proliferation measured by SRB assay. ΔA490: Change in absorbance at 490nm compared to cells prior to treatment. Results are mean ± SEM of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ difference in comparison to 0 by ANOVA followed by least significant difference test. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ determined by Student's t-test.

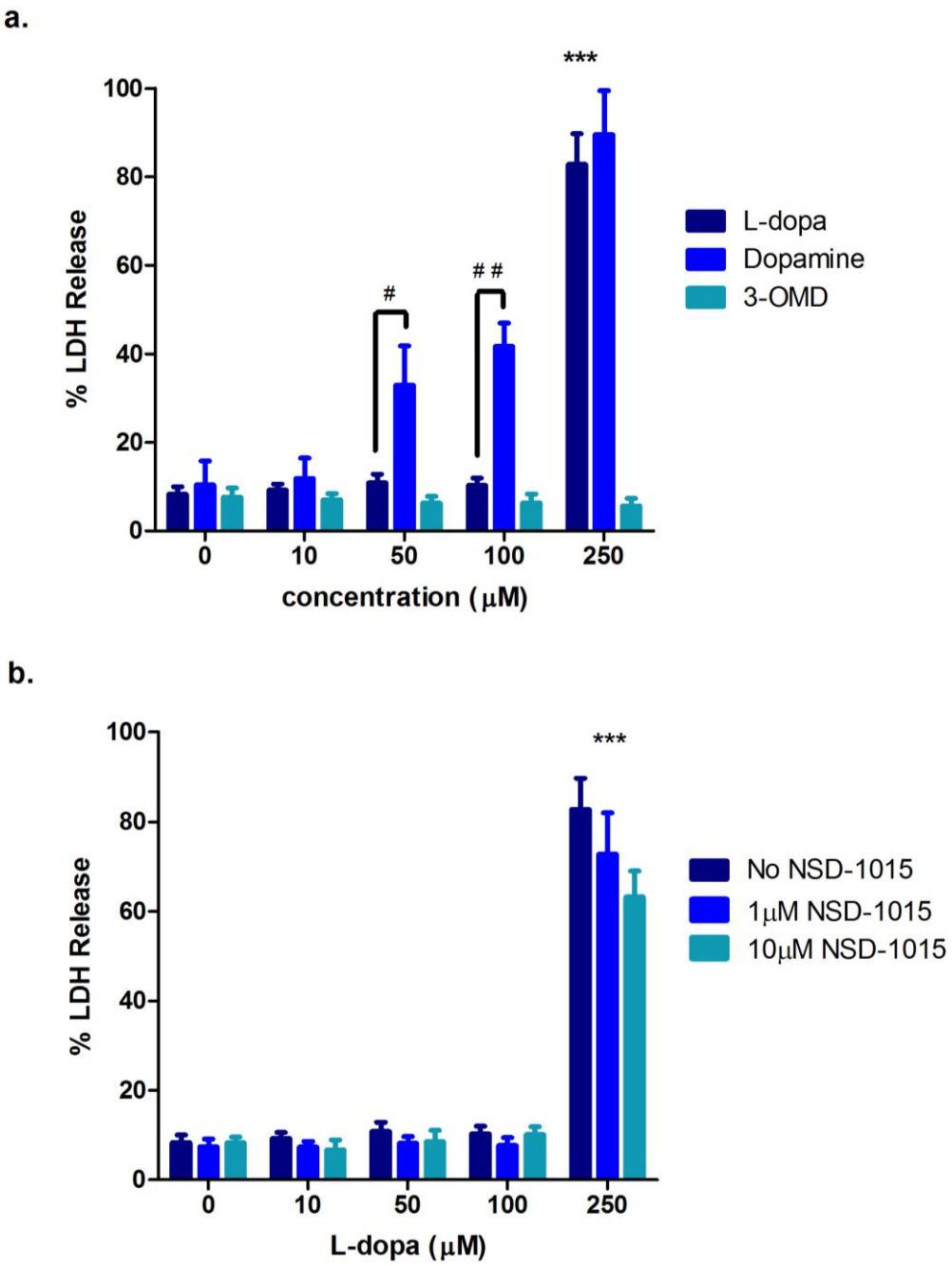


Figure 5.2 SH-SY5Y LDH release following L-dopa, dopamine, 3-OMD and NSD-1015 treatment. a. The effect of L-dopa, dopamine and 3-OMD on membrane integrity measured by the LDH release assay. b. LDH release in response to L-dopa following treatment with NSD-1015. % LDH release: LDH activity in cell culture medium as a percentage of total LDH activity following complete cell lysis. Results are mean ± SEM of 5 independent experiments. ***p <0.001 difference in comparison to 0 by ANOVA followed by least significant difference test. $^{\#}$ p <0.05, $^{\#\#}$ p <0.01 determined by Student's t-test.

level of LDH release in response to 250µM L-dopa appeared to be reduced by NSD-1015 at 1µM and 10µM, however this effect was not statistically significant ($p = 0.391$, $p = 0.060$; see figure 5.2b).

5.5.3 Intracellular GSH neuroblastoma cells

Treatment of SH-SY5Y cells with 50µM L-dopa for 48hr led to an approximately 2-fold increase in intracellular levels of GSH compared to untreated control ($p < 0.01$; see figure 5.3a). No effect on intracellular GSH was seen at earlier time points after treatment with 50µM L-dopa. Likewise intracellular GSH was unaltered by treatment with 10µM L-dopa across all tested time points. In comparison to untreated control intracellular GSH was increased by approximately 3-fold after both 24hr and 48hr treatment with 50µM dopamine ($p < 0.05$ for both; see figure 5.3b). No increase in intracellular GSH was seen after 50µM dopamine at earlier time points. Furthermore 10µM dopamine treatment did not alter intracellular GSH across all tested time points. SH-SY5Y cells have been reported to express dopamine β-hydroxylase (Biedler *et al*, 1978) therefore it was considered possible that the effect of dopamine was due to conversion to noradrenaline. Consequently the effect of 50µM noradrenaline on intracellular GSH was also tested. This concentration of noradrenaline had no effect on intracellular GSH in SH-SY5Y cells after 48hr treatment ($p = 0.968$), indicating that the effect of dopamine was not related to conversion to noradrenaline (see figure 5.4a).

To examine whether the response of intracellular GSH to L-dopa was dependent on conversion to dopamine SH-SY5Y cells were treated with NSD-1015. Treatment with 10µM and 50µM NSD-1015 significantly inhibited the increase in intracellular GSH seen after 48hr treatment with 50µM L-dopa ($p < 0.01$, $p < 0.001$ respectively; see figure 5.4b). As inhibition of AADC was associated with an increase in 3-OMD concentrations (see table 5.1) the effect of 3-OMD upon the response of intracellular GSH to L-dopa treatment was therefore also examined. Treatment with 50µM 3-OMD alone for 48hr produced no change in intracellular GSH in SH-SY5Y cells ($p = 0.269$; see figure 5.4a). Treatment of SH-SY5Y cells with 50µM L-dopa and 50µM 3-OMD combined for 48hr led to significant increases in intracellular GSH ($p < 0.001$) and this increase was not significantly different from treatment with 50µM L-dopa alone ($p = 0.229$).

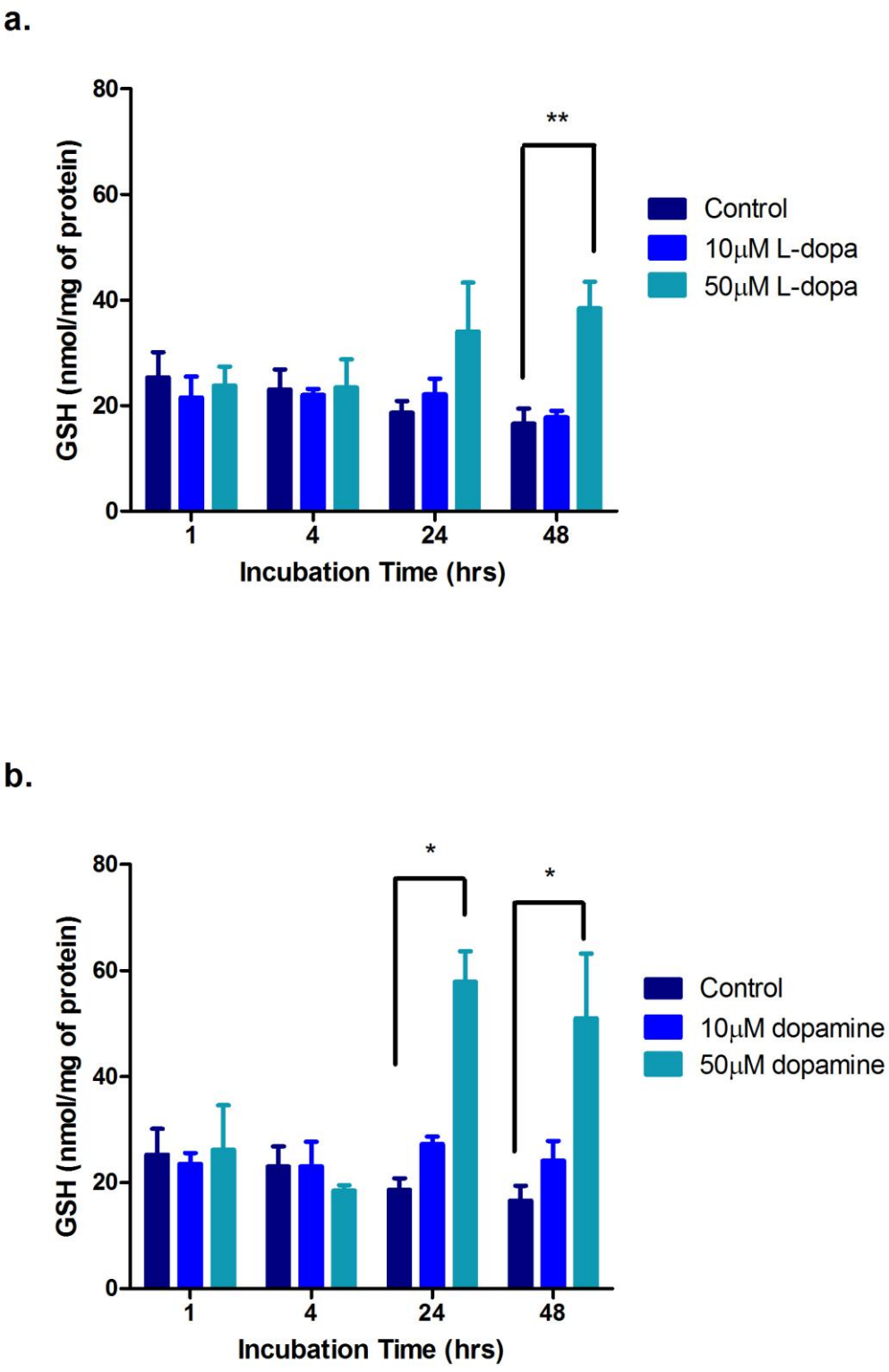


Figure 5.3 Intracellular GSH in SH-SY5Y cells following treatment with L-dopa or dopamine. L-dopa or b. dopamine. Results are mean ± SEM of 3-4 independent experiments. *p <0.05, **p <0.01 determined by ANOVA followed by least significant difference test.

To investigate the involvement of extracellular auto-oxidation in the increase in intracellular GSH in response to dopamine and L-dopa the antioxidant enzymes catalase and superoxide dismutase were added to SH-SY5Y cells 24hr before and during dopamine or L-dopa treatment. Catalase converts H_2O_2 to $2H_2O$ and superoxide dismutase converts $O_2^{\bullet-}$ to H_2O_2. The addition of 100U/mL catalase had no effect on the increase in GSH seen in SH-SY5Y cells treated with 50µM dopamine for 48hr ($p = 0.428$) or on the level of GSH measured in control cells ($p = 0.113$; see figure 5.5). However combined treatment of 100U/mL catalase with 50µM L-dopa resulted in an approximately 1.4-fold increase in intracellular GSH in comparison to cells treated with 50µM L-dopa alone ($p < 0.05$; see figure 5.5). The extracellular addition of 500U/mL superoxide dismutase also had no significant effect on the increase in intracellular GSH seen in response to 50µM dopamine or L-dopa ($p = 0.770$, $p = 0.160$ respectively), nor on the level of GSH in untreated control cells ($p = 0.737$; see figure 5.5).

The response of intracellular GSH in SH-SY5Y cells to dopamine was further characterised by inhibiting different components of the dopamine system. SH-SY5Y cells were treated with 10µM tranylcypromine, a non-selective MAO inhibitor, for 24hr before and during dopamine or L-dopa treatment. This treatment had no effect on the intracellular concentration of GSH in control cells ($p = 0.885$) or on the increased levels of GSH seen after treatment for 48hr with 50µM dopamine or L-dopa ($p = 0.395$, $p = 0.132$, respectively; see figure 5.5). To ensure that 10µM tranylcypromine treatment was inhibiting MAO the levels of catecholamines were measured after incubation with 50µM L-dopa for 48hr in SH-SY5Y cells. This treatment produced significant decreases in intracellular DOPAC and HVA ($p < 0.05$, $p < 0.01$ respectively), extracellular DOPAC and HVA ($p < 0.001$, $p < 0.01$ respectively) and a significant increase in extracellular dopamine ($p < 0.001$; see table 5.2). Intracellular DOPAC was decreased by approximately 80%, suggesting a relatively high level of MAO inhibition.

SH-SY5Y cells were also treated with the COMT inhibitor Ro-41-0960. At 10µM this treatment led to a decrease in the intracellular concentration of HVA by approximately 85% after 48hr treatment with 50µM L-dopa compared to control cells treated with L-dopa alone ($p < 0.001$; see table 5.2). Likewise extracellular HVA was undetectable after Ro-41-0960 treatment compared to a control value of 4436.34 ± 541.62nM.

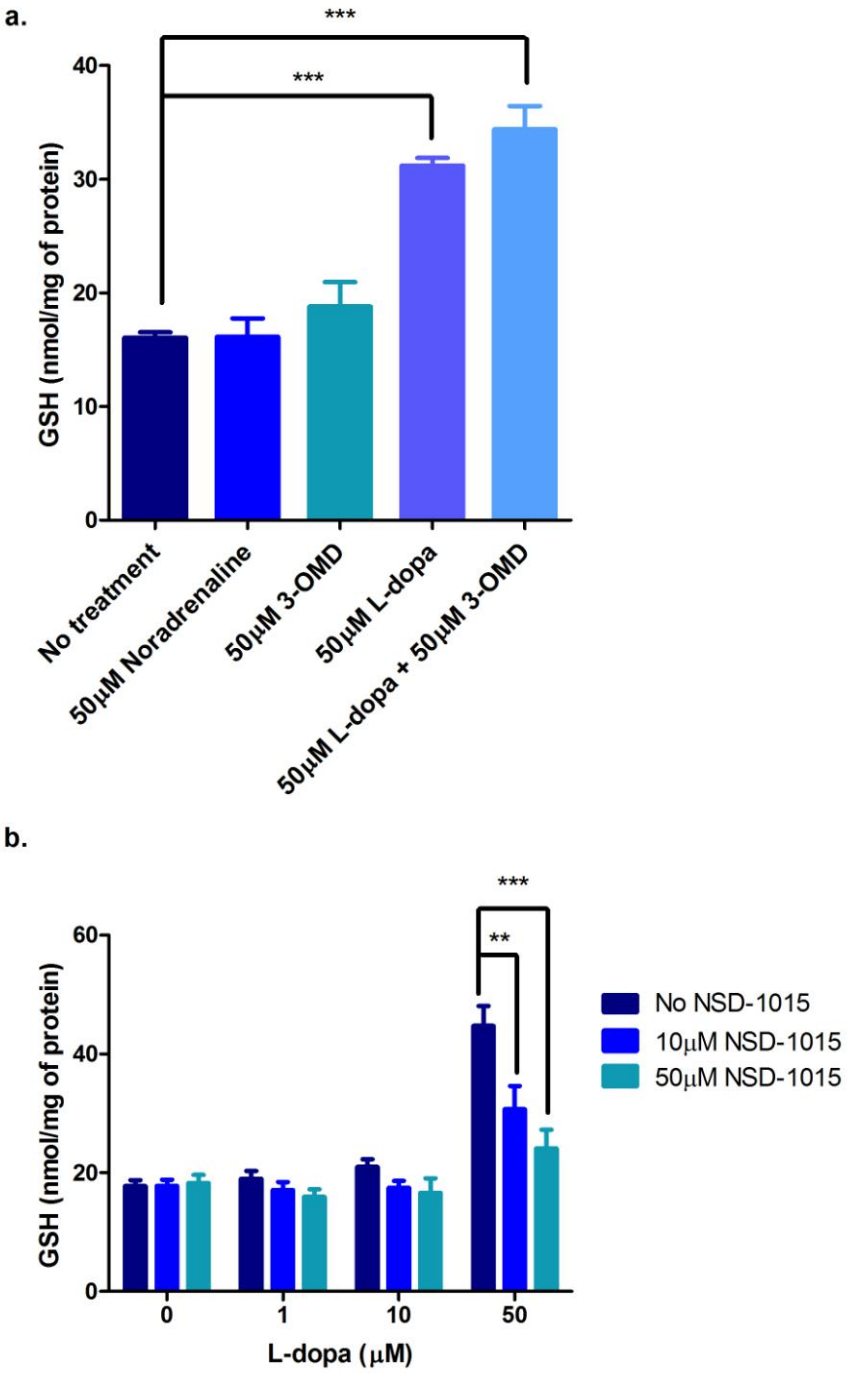


Figure 5.4 Intracellular GSH in SH-SY5Y following noradrenaline, 3-OMD and NSD-1015 treatment. a. Intracellular GSH in SH-SY5Y cells following 48hr treatment as indicated, mean ± SEM of 3-4 independent experiments b. Intracellular GSH in SH-SY5Y cells treated with NSD-1015 for 24hr and subsequently for 48hr with L-dopa, mean ± SEM of 10 independent experiments. **p <0.01, ***p <0.001 determined by ANOVA followed by least significant difference test.

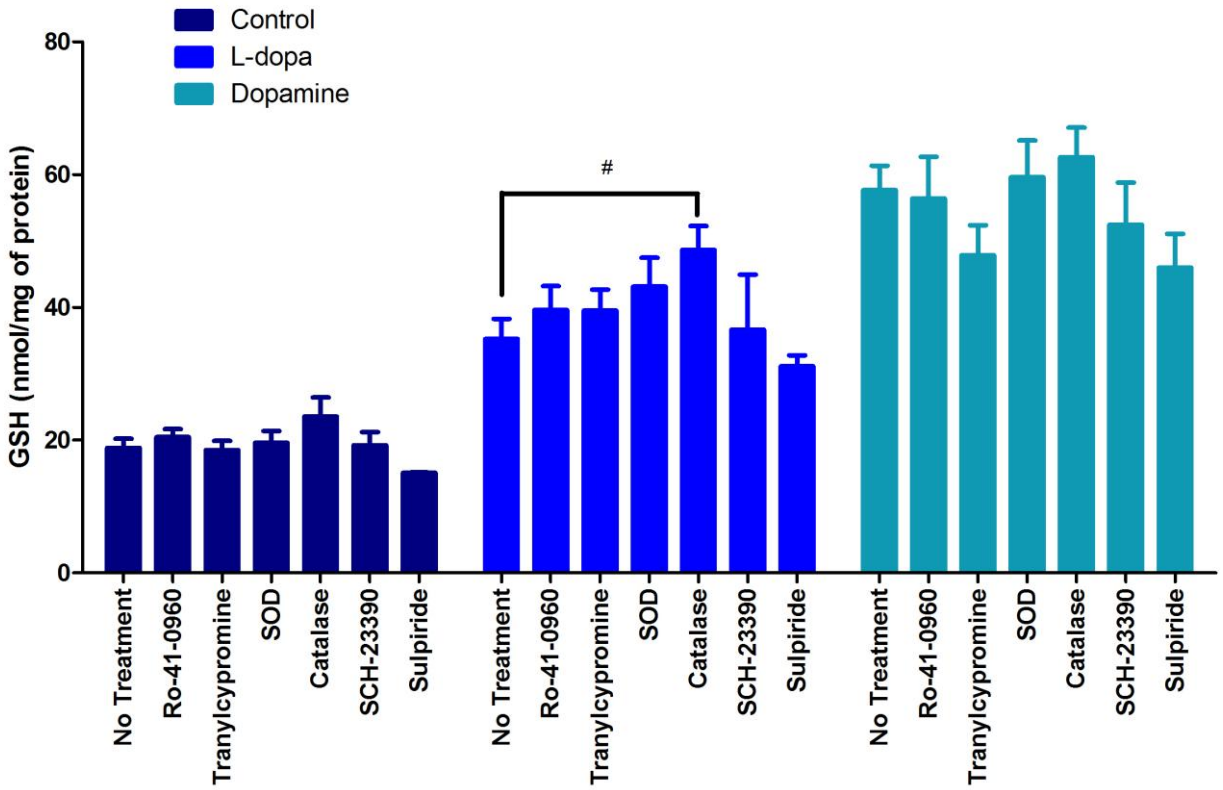


Figure 5.5 Intracellular GSH in SH-SY5Y cells following treatment with L-dopa and dopamine influence of receptors, degradation and auto-oxidation. Cells were treated with 500U/mL SOD, 100U/mL catalase, 10µM tranylcypromine or 10µM Ro-41-0960 for 24hr or with 10µM SCH-23390 or 10µM sulpiride for 1hr followed by a 48hr incubation with 50µM L-dopa or dopamine or vehicle (control). Results are mean ± SEM of 3-10 independent experiments. $^{\#}$ $p < 0.05$ determined by Student's t-test.

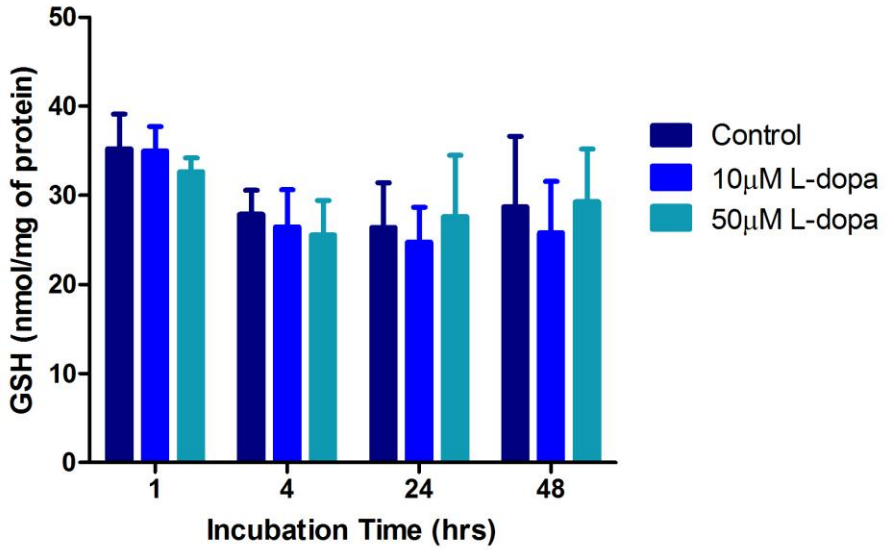
Upstream of COMT, DOPAC and dopamine were significantly increased intracellularly and dopamine increased extracellularly in Ro-41-0960 treated cells in comparison to control (see table 5.2). Together this indicates a high level of COMT inhibition by Ro-41-0960. However treatment with Ro-41-0960 did not alter the increased levels of GSH seen in cells treated with 50µM L-dopa or dopamine ($p = 0.468$, $p = 0.398$ respectively) or the GSH concentration in control cells ($p = 0.857$; see figure 5.5).

The involvement of dopamine receptors in the GSH response to dopamine and L-dopa was also investigated. Dopamine receptor antagonists were added to SH-SY5Y cultures 1hr prior to the addition of L-dopa or dopamine and then incubated for 48hr. The D1-like (D_1 and D_5) receptor antagonist SCH 23390 at 10µM had no effect on intracellular GSH concentrations in control cells ($p = 0.887$) or on the increase in GSH seen after dopamine or L-dopa treatment ($p = 0.505$, $p = 0.843$ respectively; see figure 5.5). Similarly the D2-like (D_2, D_3 and D_4) antagonist sulpiride at 10µM also had no effect upon intracellular GSH concentrations in control cells ($p = 0.179$), or after dopamine or L-dopa treatment ($p = 0.140$, $p = 0.495$ respectively).

Treatment	L-dopa	3-OMD	dopamine	DOPAC	HVA
INTRACELLULAR					
L-dopa control	21 ± 6	2 ± 2	289 ± 46	255 ± 55	171 ± 31
L-dopa + MAOI	22 ± 9	1 ± 1	294 ± 81	51 ± 13*	54 ± 24**
L-dopa +COMTI	25 ± 7	0 ± 0	476 ± 62**	966 ± 99***	25 ± 24***
EXTRACELLULAR					
L-dopa control	4689 ± 602	26 ± 26	1963 ± 200	3761 ± 311	4436 ± 542
L-dopa + MAOI	4535 ± 391	31 ± 31	5716 ± 549***	3203 ± 1535	1466 ± 441***
L-dopa +COMTI	5301 ± 640	92 ± 39	3787 ± 458**	6716 ± 1183	0 ± 0***

Table 5.2 Catecholamine levels following treatment with MAO and COMT inhibitors. 10µM tranylcyromine (MAOI) or 10µM Ro-41-0960 (COMTI) added 24hr before the addition of 50µM L-dopa. Intracellular values expressed as pmol/mg of protein and extracellular values expressed as nM. All values are mean ± SEM of 4-5 independent experiments. *p <0.05, **p <0.01, ***p <0.001 difference from control determined by ANOVA followed by least significant difference test.

a.



b.

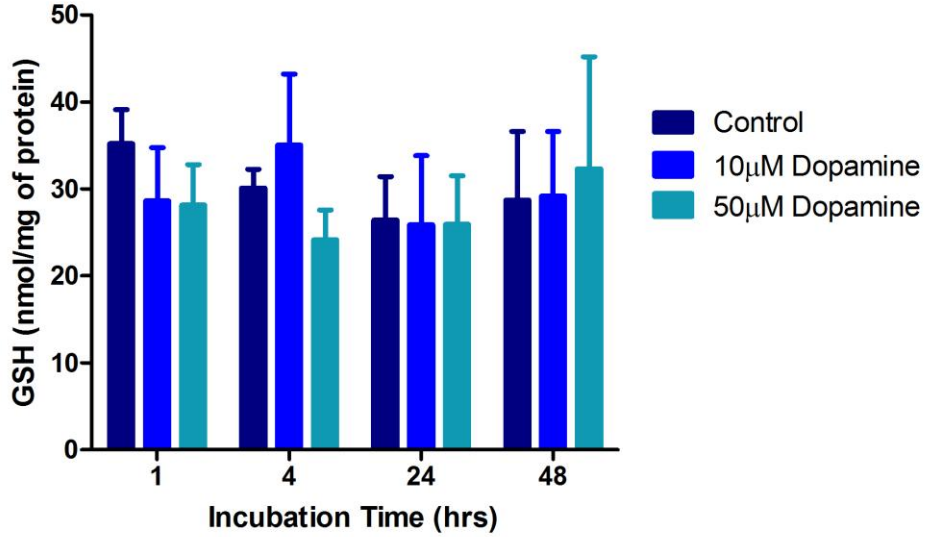


Figure 5.6 Intracellular GSH in 1321N1 cells following treatment with L-dopa and dopamine. a. L-dopa or b. dopamine. Results are mean ± SEM of 3-4 independent experiments.

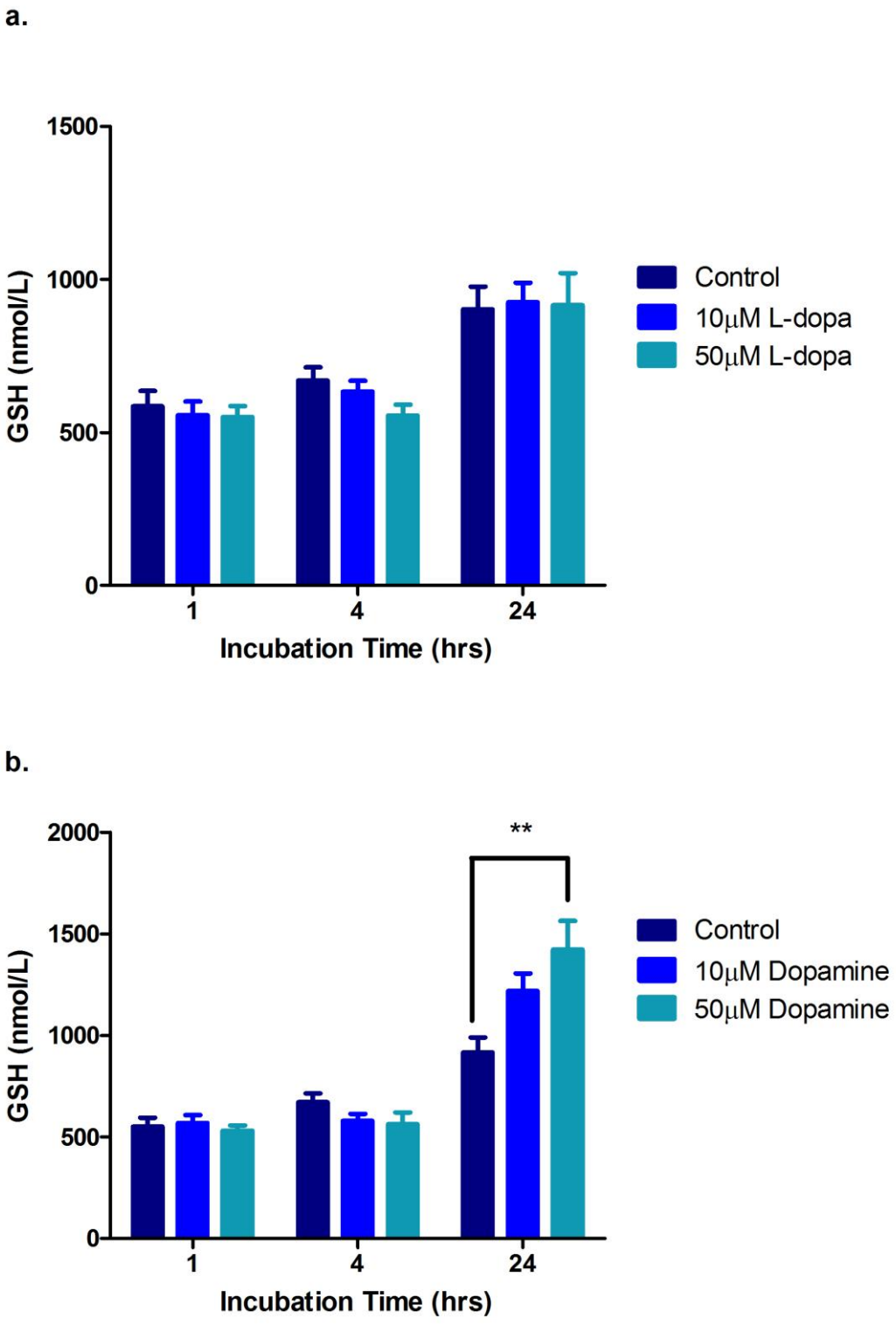


Figure 5.7 GSH release from 1321N1 cells following L-dopa or dopamine treatment. GSH release measured over 4hr following incubation with a. L-dopa or b. dopamine. All results are mean ± SEM from 7-8 independent experiments. **p <0.01 determined by ANOVA followed by least significant difference test.

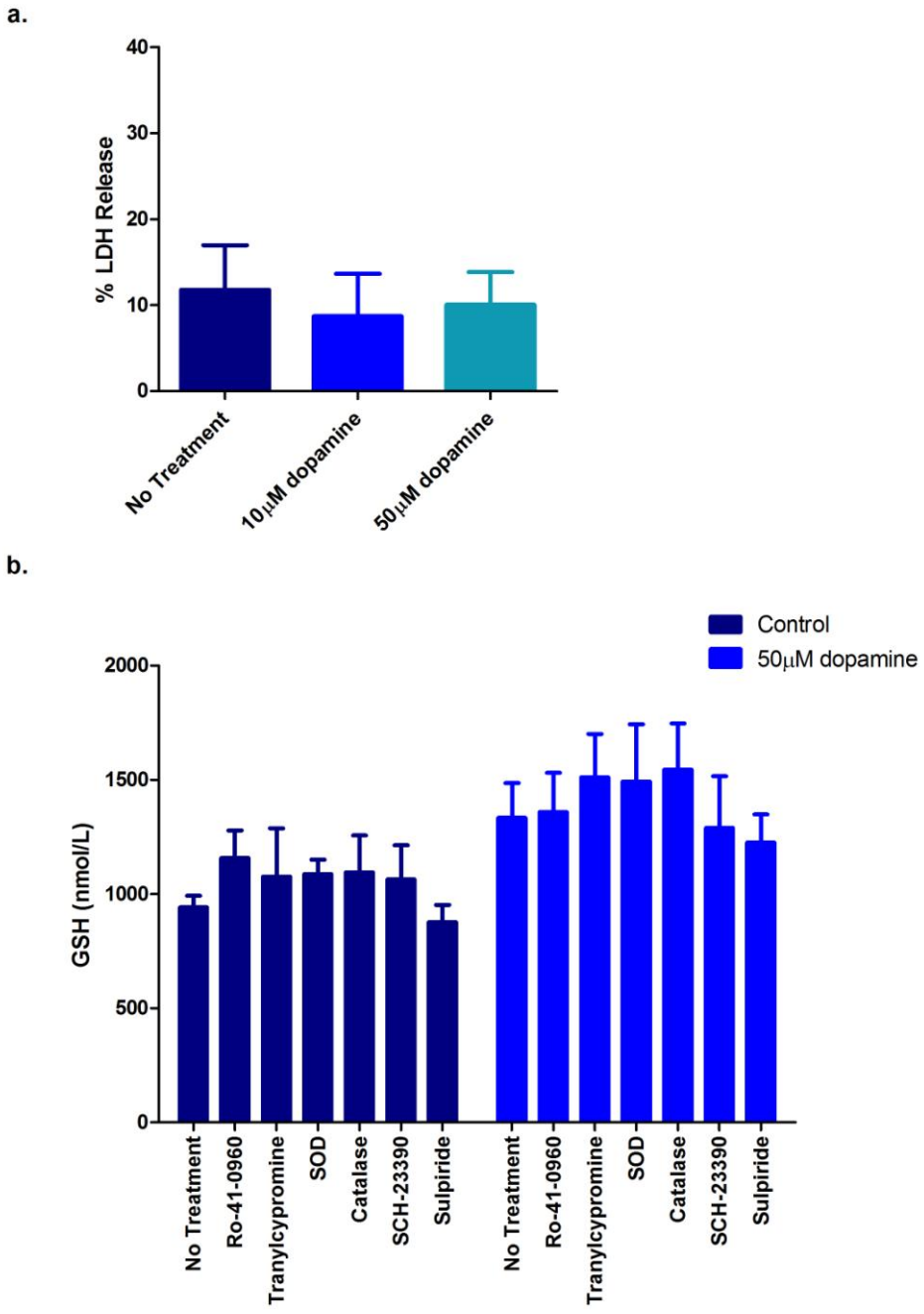


Figure 5.8 LDH and GSH release from 1321N1 cells following dopamine treatment influence of receptors, degradation and auto-oxidation. a. LDH release from 1321N1 cells over 4hr in minimal medium following pre-treatment with dopamine for 24hr, mean ± SEM from 3-4 independent experiments. b. GSH release from 1321N1 cells. Cells were treated with 500U/mL SOD, 100U/mL catalase, 10µM tranylcypromine or 10µM Ro-41-0960 for 24hr or with 10µM SCH-23390 or 10µM sulpiride followed by a 24hr incubation with 50µM dopamine or vehicle (control). Results are mean ± SEM from 3-4 independent experiments.

5.5.4 Intracellular GSH astrocytoma cells

The effect of L-dopa and dopamine on intracellular GSH concentration was also investigated in the human astrocytoma cell line 1321N1. L-dopa treatment at either 10µM or 50µM for up to 48hr had no effect on intracellular GSH concentrations (see figure 5.6a). Similarly 10µM or 50µM dopamine also had no effect on intracellular GSH levels for up to 48hr of treatment (see figure 5.6b). One important difference between astrocytes and neurons is that astrocytes have an approximately 2-fold higher GSH content and consequently a higher antioxidant capacity (Makar *et al*, 1994; Bolanos *et al*, 1995). Untreated 1321N1 astrocytoma cells also had an approximately 1.8-fold higher level of intracellular GSH in comparison to untreated SH-SY5Y cells in the current study at 72hr in culture ($p = 0.01$, $n = 4$).

5.5.5 GSH Release from human astrocytoma cells

The trafficking of GSH from astrocytes to neurons is considered to be an important mechanism for maintaining neuronal GSH levels (see section 1.7.6). The first stage in the trafficking process is the release of GSH from astocytes. The effect of L-dopa and dopamine on the release of GSH from astrocytomas was consequently investigated. Cells were treated with L-dopa or dopamine and subsequently washed and incubated for 4hr in minimal medium. The amount of GSH released was defined as the concentration of GSH in the minimal medium after this 4hr period. L-dopa treatment for up to 24hr had no effect on GSH release from astrocytoma cells at either 10µM or 50µM ($p = 0.609$; see figure 5.7a). Conversely treatment of astrocytoma cells with dopamine for 24hr led to significant increases in GSH release with 50µM dopamine ($p < 0.05$, $p < 0.01$ respectively; see figure 5.7b). It is important to note that the 1321N1 astrocytoma cells were not found to express detectable AADC activity with L-dopa as substrate (see section 4.5.1), consequently added L-dopa would not be converted to dopamine in this cell line. To determine whether the release of GSH was specific or whether it was related to disruption of membrane integrity LDH release into the minimal media over the 4hr incubation was measured after 24hr treatment with dopamine in the same manner as GSH release measurement. No significant change in LDH release was found for dopamine treatment at 10µM or 50µM in comparison to untreated control ($p = 0.693$, $p = 0.797$ respectively; see figure 5.8a). This indicates that dopamine treatment induced a specific increase in GSH release from astrocytoma cells, rather than a non-specific effect on cell lysis.

Treatment with 100U/mL catalase for 24hr prior and during the 24hr incubation with 50µM dopamine produced no change in the increased level of GSH release from astrocytoma cells ($p = 0.430$; see figure 5.8b). This treatment also did not change the level of GSH release from untreated control cells ($p = 0.350$). Likewise 500U/mL superoxide dismutase had no effect on GSH release in control cells ($p = 0.130$) or on the increased release from dopamine treated cells ($p = 0.591$; see figure 5.8b). Treatment with 10µM tranylcypromine or Ro-41-0960 for 24hr prior to and during the incubation with 50µM dopamine had no effect on the increased amount of GSH release ($p = 0.492$, $p = 0.916$ respectively; see figure 5.8b). The level of GSH release from untreated control cells was also unchanged by treatment with 10µM tranylcypromine or Ro-41-0960 ($p = 0.505$, $p = 0.128$ respectively). Treatment with 10µM of the dopamine receptor antagonists SCH-23390 or sulpiride for 1hr before and during incubation with 50µM dopamine did not alter the increased GSH release from astrocytoma cells ($p = 0.874$, $p = 0.627$, respectively; see figure 5.8b). 10µM SCH 23390 or sulpiride also did not change GSH release from untreated control astrocytoma cells ($p = 0.420$, $p = 0.489$ respectively).

5.5.6 GSH Release from human neuroblastoma cells

GSH release was measured in the same way as for 1321N1 cells (see section 5.5.5). Treatment of SH-SY5Y cells with 50µM dopamine for 48hr produced an approximately 5-fold increase in GSH release ($p < 0.01$, see figure 5.9a). However this treatment also resulted in an approximately 2.5-fold increase in LDH release ($p < 0.01$, figure 5.9b), indicating that the effect of dopamine on GSH release was non-specific, and potentially as a result of increased cell lysis. 48hr treatment with 50µM L-dopa had no significant effect on either GSH release or LDH release over the 4hr incubation in minimal medium ($p = 0.776$, $p = 0.064$ respectively; see figure 5.9). 50µM L-dopa treatment for 48hr in the presence of 10µM NSD-1015 also had no significant effect on GSH release or LDH release over the 4hr incubation in minimal medium ($p = 0.569$, $p = 0.244$, respectively; see figure 5.9).

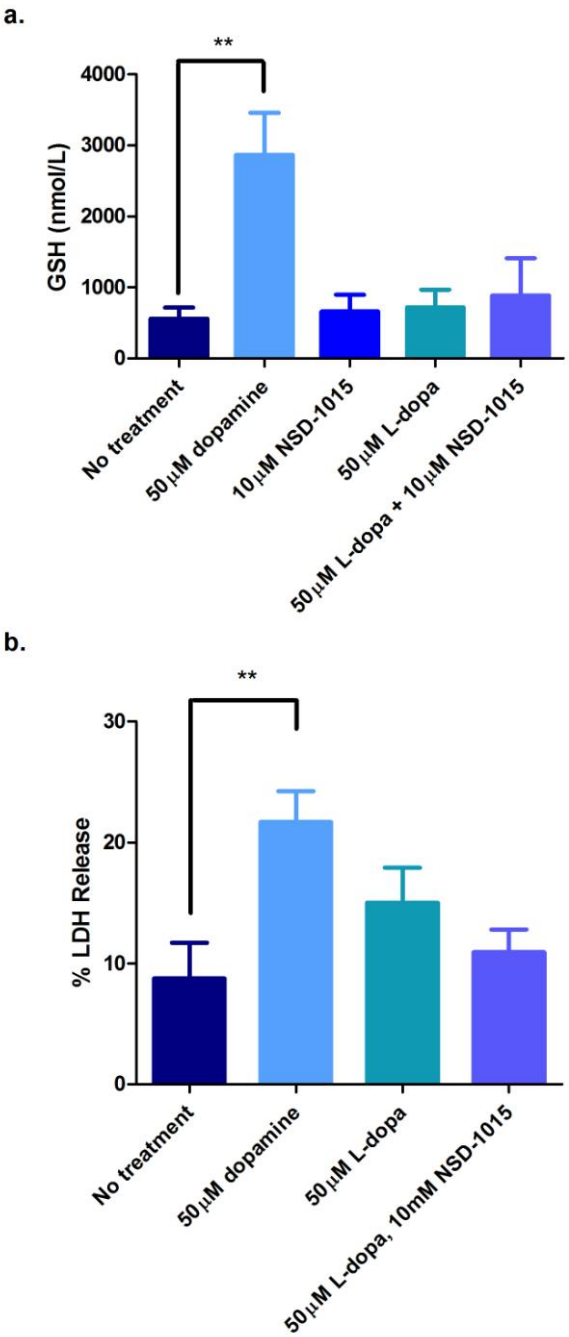


Figure 5.9 GSH and LDH release from SH-SY5Y cells following treatment with L-dopa and dopamine. a. GSH release over 4hr from SH-SY5Y cells following incubation for 48hr with treatment as indicated, NSD-1015 was added 24hr before other treatments, mean ± SEM from 3 independent experiments. LDH release from SH-SY5Y cells over 4hr in minimal medium following pre-treatment as indicated for 48hr, NSD-1015 was added 24hr before other treatments, mean ± SEM from 4 independent experiments. **p <0.01 determined by ANOVA followed by least significant difference test.

5.5.7 Mitochondrial respiratory chain

As 50µM dopamine and L-dopa have effects on cellular viability and 50µM dopamine was found to influence GSH concentrations in SH-SY5Y cells and therefore the effects of 48hr treatment with 50µM dopamine or L-dopa on the activity of the mitochondrial respiratory chain in SH-SY5Y cells was also investigated. 50µM L-dopa had no significant effect on complex I, II/III or IV either in the presence or absence of 10µM NSD-1015 (see figure 5.10). 10µM NSD-1015 alone also had no significant effect on any of the mitochondrial respiratory chain complexes. 50µM dopamine had no significant effect on the activity of complex II/III or IV, however this treatment led to an approximately 2-fold increase in the activity of complex I (see figure 5.10a, table 5.3). This effect was apparent both when complex I activity was expressed as a ratio to citrate synthase or as nmol/min/mg of protein. Furthermore dopamine did not significantly change the % rotenone inhibition ($p = 0.530$), indicating that the increase in complex I activity was not related to changes in non-specific NADH oxidation.

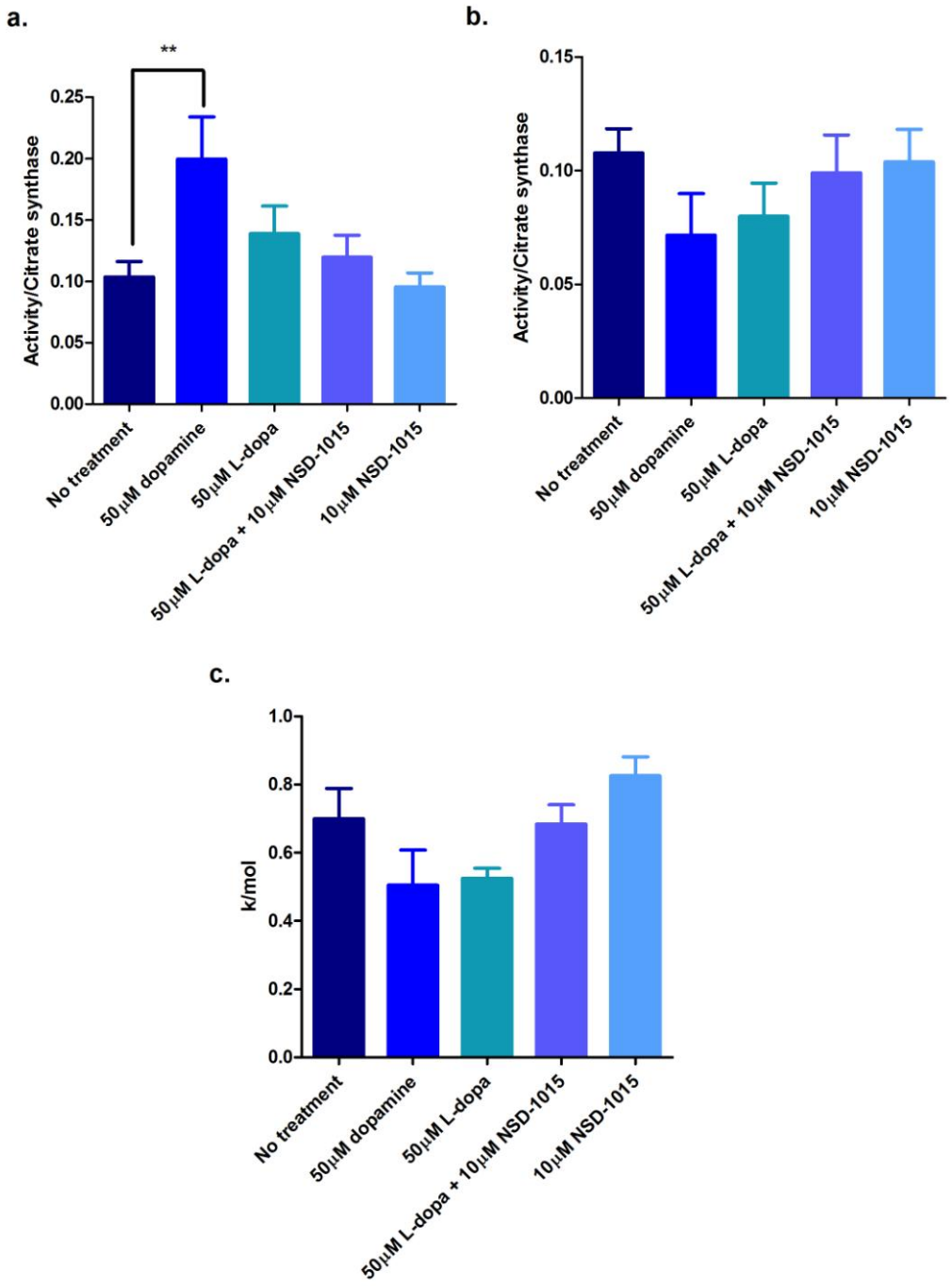


Figure 5.10 SH-SY5Y cells activity of mitochondrial respiratory chain following treatment with L-dopa, dopamine and NSD-1015. Activity of a. complex I, b. complex II/III and c. complex IV expressed as a ratio to citrate synthase. Cells were treated as indicated for 48hr, NSD-1015 was added 24hr before other treatments. Results are mean ± SEM from 4 independent experiments. ** $p < 0.01$ determined by ANOVA followed by least significant difference test.

	Complex I		Complex II/III		Complex IV	Citrate synthase
	Activity	% inhibition	Activity	% inhibition		
Control	10.6 ± 1.8	71.3 ± 2.2	11.24 ± 2.3	96.5 ± 0.4	69.8 ± 7.0	103.0 ± 14.1
Dopamine	20.4 ± 4.2**	68.1 ± 1.9	7.4 ± 1.7	94.5 ± 1.3	51.6 ± 9.6	109.1 ± 21.1
L-dopa	15.1 ± 3.5	67.4 ± 1.4	8.2 ± 1.9	98.7 ± 1.7	54.0 ± 5.9	105.2 ± 16.3
L-dopa + NSD-1015	12.0 ± 1.2	75.9 ± 5.6	10.0 ± 1.2	96.6 ± 1.2	70.1 ± 5.9	103.5 ± 9.1
NSD-1015	7.2 ± 0.3	72.1 ± 2.6	7.9 ± 1.0	97.7 ± 1.2	63.3 ± 8.7	76.4 ± 2.4

Table 5.3 SH-SY5Y cells activity of mitochondrial respiratory chain following treatment with L-dopa, dopamine and NSD-1015. SH-SY5Y cells activity of mitochondrial respiratory chain complex I (nmol/min/mg of protein), complex II/III (nmol/min/mg of protein) and complex IV (k/min/mg of protein) as well as citrate synthase activity (nmol/min/mg). Complex I % inhibition following addition of rotenone and complex II/III % inhibition following addition of antimycin A. Cells were treated as indicated for 48hr, 10µM NSD-1015 was added 24hr before other treatments. Dopamine and L-dopa were added at a concentration of 50µM. Results are mean ± SEM from 4 independent experiments. ** $p < 0.01$ determined by ANOVA followed by least significant difference test.

5.6 Discussion

5.6.1 The use of NSD-1015 to model AADC deficiency

NSD-1015 was used in this study to inhibit AADC activity in SH-SY5Y neuroblastoma cells. The pattern of metabolites in NSD-1015 treated cells after addition of L-dopa was similar to that seen in the CSF of AADC deficient patients (Hyland and Clayton, 1990; Brun *et al*, 2010). Adding dopamine to the SH-SY5Y cells instead of L-dopa should bypass the NSD-1015 inhibition of AADC. It would therefore be expected that dopamine metabolites would be at similar levels in cells treated with NSD-1015 and dopamine in comparison to cells treated with dopamine alone. However the results presented here demonstrated that this inhibitor led to decreased DOPAC, HVA and 3-OMD following the addition of dopamine in comparison to cells treated only with dopamine. The decreased levels of 3-OMD following dopamine and NSD-1015 treatment could indicate direct inhibition of COMT, however this decrease in 3-OMD may also be explained by competition between L-dopa and dopamine for COMT. The dopamine NSD-1015 treated cells had higher levels of dopamine in comparison to cells treated only with dopamine. This raised dopamine may have increased dopamine metabolism via COMT, increasing competition with L-dopa for COMT, consequently decreasing 3-OMD production. Furthermore the cells treated with L-dopa and NSD-1015 had raised levels of 3-OMD as would be expected from AADC inhibition leading to increased degradation of L-dopa via COMT, but also indicating that NSD-1015 does not lead to direct inhibition of COMT. The decrease in DOPAC and HVA levels in dopamine and NSD-1015 treated cells could indicate that NSD-1015 can directly inhibit MAO. However it is also possible that inhibition or deficiency of AADC leads to cellular alterations which may decrease dopamine degradation via MAO. For example AADC can produce not only dopamine and serotonin but also trace amines such as 2-phenylethylamine and tyramine which are known to influence dopamine neurotransmission (reviewed by Burchett and Hicks, 2006). It is possible therefore that the inhibition of AADC could lead to downregulation of MAO, potentially through decreased levels of trace amines, leading to the decreased levels of DOPAC and HVA observed following dopamine and NSD-1015 treatment. Further work may be required using an AADC knockdown approach to determine whether AADC deficiency can influence MAO expression or activity.

5.6.2 Catecholamine toxicity in SH-SY5Y cells

This study demonstrated both cell loss and increased LDH release in response to L-dopa or dopamine at concentrations greater than 250µM. This observation has been demonstrated before in SH-SY5Y cells and in a range of other cell types (Mena *et al*, 1993; Basma *et al*, 1995; Lai and Yu, 1997; Nakao *et al*, 1997; Offen *et al*, 2001; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003). Cell death has been correlated with the formation of quinones and melanin indicating auto-oxidation of L-dopa and dopamine was involved in toxicity (Pardo *et al*, 1995; Basma *et al*, 1995; Lai and Yu, 1997). The addition of antioxidants including ascorbic acid, GSH, sodium metabisulphite and N-acetyl-L-cysteine have all protected cells to some extent from L-dopa and dopamine induced cell death (Pardo *et al*, 1995; Lai and Yu, 1997; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003). Furthermore extracellular addition of the enzymes catalase and superoxide dismutase also decreased L-dopa and dopamine induced cell death in most experiments demonstrating that $O_2^{\bullet-}$ and H_2O_2 contribute to toxicity (Basma *et al*, 1995; Lai and Yu, 1997; Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003). Induction of apoptosis was also found to be involved in the mechanism of L-dopa and dopamine induced cell death (Pedrosa and Soares-Da-Silva, 2002; Haque *et al*, 2003).

In the present study dopamine was demonstrated to increase LDH release at 50µM and 100µM, whilst L-dopa had no effect at these concentrations. This may suggest some difference in the mechanism of L-dopa and dopamine toxicity such that dopamine affects membrane integrity to a greater extent than L-dopa. One potential explanation for this disparity could relate to an interaction between dopamine and lipids for example dopamine is known to form adducts with malondialdehyde, a product of lipid peroxidation (d'Ischia *et al*, 1993; d'Ischia *et al*, 1995). Lai and Yu (1997) observed an increase in lipid peroxidation in SH-SY5Y cells after 10µM dopamine treatment but a decrease at >100µM. The authors suggested that as the study measured thiobarbituric acid (TBA) reactivity with malondialdehyde the apparent decrease in lipid peroxidation may instead indicate formation of dopamine-malondialdehyde adducts which may not be reactive with TBA (d'Ischia *et al*, 1993; d'Ischia *et al*, 1995; Lai and Yu, 1997). Spencer *et al* (1996) using the same TBA reactivity method suggested that dopamine and L-dopa inhibit lipid peroxidation, and that dopamine was the more potent inhibitor. However as the TCA method may have been measuring malondialdehyde adduct

formation, rather than inhibition of lipid peroxidation, then this observation could instead be demonstrating that dopamine forms adducts with malondialdehyde more readily than L-dopa. Whether dopamine-adduct formation with malondialdehyde effects membrane integrity has not been investigated, however Lai and Yu (1997) also demonstrated an increase in LDH release from SH-SY5Y cells at the same dopamine concentrations that were suggested to increase adduct formation.

3-OMD had no effect on SH-SY5Y cell proliferation or on LDH release in the current study. This indicates that 3-OMD is non-toxic, which has also been demonstrated in previous investigations (Offen *et al*, 2001). The lack of toxicity most likely relates to the non-oxidisable nature of 3-OMD due to the presence of the methoxy group at position 4 of the phenyl ring (Offen *et al*, 2001; Haque *et al*, 2003). This could suggest that the methylation of L-dopa to 3-OMD by COMT may be a protective mechanism against L-dopa auto-oxidation. In support of this the extracellular addition of purified COMT was demonstrated to have a small protective effect on L-dopa induced toxicity in PC12 cells (Offen *et al*, 2001). In the CSF of AADC deficient patients the level of 3-OMD is generally higher than that of L-dopa (Brun *et al*, 2010). This indicates that much of the L-dopa produced by AADC deficient patients is converted to 3-OMD. This methylation may decrease the half-life of L-dopa in the brain and consequently may reduce the possibility of auto-oxidation occurring. In AADC deficiency COMT inhibition could be considered as a treatment to prevent the breakdown of dopamine, however if COMT acts to protect against L-dopa toxicity then the use of COMT inhibitors may be detrimental to AADC deficient patients. COMT inhibitors are currently used to treat Parkinson's disease to prevent peripheral methylation of administered L-dopa, but they may also inhibit central COMT (Ceravolo *et al*, 2002; Factor, 2008). COMT inhibitors have also been used in the treatment of disorders of BH_4 metabolism (Pearl et al, 2007). However it has been reported that inhibition of COMT in mesencephalic cultures protects against L-dopa toxicity by a glial dependent mechanism (Storch *et al*, 2000; Blessing *et al*, 2003). It may be important therefore to examine the effect of COMT inhibitors on L-dopa induced toxicity in an *in vivo* model of AADC deficiency.

It is important to note that the concentration of L-dopa required to induce toxicity in SH-SY5Y cells (250µM) is much higher than that seen in the CSF of AADC deficient patients (Patient Range: <2-549 nM). The concentration of L-dopa is likely to be higher

in the vicinity of tyrosine hydroxylase containing neurons than in the spinal cord, but may still not reach the high concentrations required for toxicity *in vitro*. Due to the use of L-dopa in the treatment of Parkinson's disease the potential neurotoxicity of L-dopa has been widely debated. The majority of *in vivo* studies have not found administration of L-dopa to be neurotoxic (Hefti *et al*, 1981; Perry *et al*, 1984; Quinn *et al*, 1986; Murer *et al*, 1998; Zeng *et al*, 2001; Mytilineou *et al*, 1993; Mytilineou *et al*, 2003; Fahn et al, 2005). Conversely in one study where high dose L-dopa was administered intraperitoneally in rat there was damage to dopaminergic neurons (Jeon *et al*, 2007). Furthermore microdialysis of substantia nigra in rat with L-dopa was found to increase the production of $OH^{\bullet-}$ (Smith *et al*, 1994).

There is potentially an important difference between L-dopa treatment of non-AADC deficient patients and the accumulation of L-dopa seen in AADC deficiency. Namely in AADC deficiency L-dopa is produced within tyrosine hydroxylase positive neurons, however the majority cannot be converted to dopamine and so may not be sequestered into vesicles potentially leading to a cytosolic accumulation of L-dopa. Certainly intracellular L-dopa was significantly increased in AADC inhibitor treated neuroblastoma cells in this study. Conversely L-dopa exogenously administered to non-AADC deficient individuals, taken up by dopaminergic neurons would then be converted into dopamine and stored in vesicles. It has been demonstrated that increased cytosolic dopamine as a result of inadequate storage can lead to neurodegeneration in rodents (Caudle *et al*, 2007; Chen *et al*, 2008a). If L-dopa is similarly toxic to dopamine, then increased cytosolic L-dopa may have a similar effect. In the current study dopamine was found to affect membrane integrity at lower concentrations than L-dopa. Similarly at concentrations <25μM dopamine had an inhibitory effect on SH-SY5Y cells in comparison to L-dopa. However at higher concentrations both affected cell proliferation to a similar extent suggesting a relatively equivalent neurotoxicity, which has also been suggested in previous studies (Lai and Yu, 1997; Pedrosa and Soares-Da-Silva, 2002). Moreover the AADC inhibitor NSD-1015 had only a minor influence on the effect of L-dopa on cell proliferation demonstrating that conversion to dopamine was not required for toxicity. The transduction of an AADC adeno-associated virus vector into primary rat neuronal cultures has been shown to protect against L-dopa induced toxicity (Doroudchi *et al*, 2005). The protection was linked to increased expression of AADC which in turn also led to increased VMAT2 expression, indicating that conversion of L-dopa to dopamine and its subsequent storage is a

protective mechanism. This mechanism would be absent in AADC deficiency and therefore potentially increased neuronal cytosolic L-dopa could be a damaging factor in this disorder. Further investigations in an *in vivo* model of AADC deficiency would be required to investigate this hypothesis.

5.6.3 Dopamine and GSH

Dopamine was demonstrated in this study to increase intracellular GSH in neuroblastoma cells and GSH release from astrocytoma cells. L-dopa treatment also led to increased intracellular GSH in neuroblastoma cells however when AADC was inhibited with NSD-1015 this effect was attenuated, suggesting conversion of L-dopa to dopamine was required for this effect. The MAO inhibitor tranylcypromine had no effect on the L-dopa induced GSH increase in neuroblastoma cells. This demonstrates that the antagonism of the L-dopa effect on GSH by NSD-1015 was likely to be related to the inhibition of AADC rather than to potential changes in MAO activity or expression as a result of NSD-1015 treatment (see section 5.6.1). L-dopa had no effect on the release of GSH from astrocytomas, in which AADC activity was undetectable and consequently L-dopa would not be converted to dopamine. Previous studies have found that L-dopa treatment can increase intracellular GSH concentrations in primary mesencephalic cultures, Neuro-2A neuroblastoma cells and pig kidney epithelial (PKE) cells (Mytilineou *et al*, 1993; Han *et al*, 1996). The inhibition of AADC by carbidopa or NSD-1055 did not alter this effect in Neuro-2A or PKE cells, moreover AADC inhibition further increased intracellular GSH levels above L-dopa treatment alone (Han *et al*, 1996). The reason for the disparity between this and the current study may relate to differences between cell-type. The increase in intracellular GSH in response to L-dopa was much lower, <1.38-fold (Han *et al*, 1996) than the 2-fold increase observed in the current study. Additionally the concentration of L-dopa used to evoke the response may also have influenced the dependence on conversion to dopamine, Han *et al* (1996) used 200µM L-dopa, 4-fold greater than that used in the current study. Taken together this could indicate that the GSH response to L-dopa reported by Han *et al* (1996) was relatively non-specific and could relate to extracellular oxidation of L-dopa generating ROS and consequently leading to a cellular response increasing intracellular GSH concentrations.

Dopamine has also been reported to induce increases in intracellular GSH in primary mesencephalic cultures and PC12 cells (Han *et al*, 1996; Jia *et al*, 2008a). The

concentration range of dopamine that elicited this response in a dose-dependent manner was between 50µM and 150µM, comparable to the present study. The increase in GSH in PC12 cells was accompanied by an increase in γ-glutamylcysteine ligase, the rate limiting enzyme in GSH synthesis (Jia *et al*, 2008a). Furthermore the activity of glutathione peroxidase and glutathione reductase were unaltered, together these observations indicate that dopamine induces an increase in *de novo* synthesis of GSH rather than increased recycling. Pre-treatment with L-dopa or dopamine, over the timescale required to induce increases in GSH, has also been demonstrated to protect cells from subsequent treatment with inducers of oxidative stress including 6-hydroxydopamine and tert-butyl hydroperoxide (Han *et al*, 1996; Jia *et al*, 2008a). Jia *et al* (2008a) demonstrated that dopamine treatment increased NAD(P)H: quinone oxidoreductase 1 (NQO1) activity and expression. NQO1 catalyses the 2-electron reduction of quinones to hydroquinones including the reduction of cyclised dopamine-o-quinone, a product of dopamine oxidation (Segura-Aguilar and Lind, 1989). Overexpression of NQO1 has been demonstrated to protect aganist dopamine-induced cellular toxicity (Zafar *et al*, 2006). A range of other chemicals including tert-butylhydroquinone, an inducer of oxidative stress, have been demonstrated to exhibit similar effects on GSH and NQO1 (Hara *et al*, 2003; Jia *et al*, 2008b; Jia *et al*, 2008c). Both γ-glutamylcysteine ligase and NQO1 have been demonstrated to be regulated by the transcription factor Nrf2 (Venugopal *et al*, 1996; Wild *et al*, 1999). Nrf2 in turn is regulated in part by Keap1 that may act as a sensor for ROS and electrophiles (reviewed by Motohashi and Yamamoto, 2004). Dopamine has previously been demonstrated to activate this pathway in a range of cell types (Shih *et al*, 2007; Wang *et al*, 2010b). It is possible therefore that the increase in intracellular GSH observed in SH-SY5Y cells and previously in PC12 cells was related to the activation of this signalling pathway (Jia *et al*, 2008a).

The effect of dopamine on release of glutathione from astrocytes has been previously investigated (Hirrlinger *et al*, 2002). In this study the authors found that incubation of primary astrocytes with 50µM dopamine decreased total glutathione (GSH + 2 x GSSG) release. This is in contrast to the present study where incubation with 10µM or 50µM dopamine increased GSH release from astrocytomas. It is possible that this may relate to a difference between astrocytoma cells and primary astrocytes. However a more likely explanation relates to the method by which the cells were treated with dopamine. In the current study astrocytoma cells were pre-treated with dopamine for 24hr, the cells

were then washed and subsequently incubated in minimal medium for 4hr and GSH was measured in the minimal medium after the 4hr. Consequently no dopamine was present during the period in which GSH release was measured. In contrast Hirrlinger *et al* (2002) incubated primary astocytes with dopamine for 6hr in minimal medium and subsequently measured total glutathione and GSSG in this medium. The first disparity is the length of dopamine treatment. In the present set of experiments 24hr was required to observe an increase in GSH release, after 4hr dopamine treatment there was no difference from control. The second disparity relates to the presence of dopamine during the period of measured glutathione release. Dopamine auto-oxidises readily in cell culture medium leading to the production of ROS (Clement *et al*, 2002). Hirrlinger *et al* (2002) demonstrated that the decrease in total glutathione in the minimal medium was prevented by the addition of extracellular SOD indicating that this process was dependent on extracellular $O_2^{\bullet-}$. Consequently the decrease in total glutathione was likely to be related to extracellular oxidation of GSH, additionally whilst GSSG was not increased following 50μM dopamine treatment the proportion of GSSG to GSH was increased further suggesting GSH oxidation (Hirrlinger *et al*, 2002). Therefore in the study of Hirrlinger and colleagues the loss of extracellular total glutathione may potentially relate to GSH-adduct formation rather than a decrease in release of GSH from astrocytes.

The role of extracellular auto-oxidation in the dopamine induced increases in GSH within neuroblastomas and GSH release from astrocytomas was investigated by adding the antioxidant enzymes catalase and SOD to the cell culture medium. As neither catalase nor SOD inhibited the rise in GSH in neuroblastomas or release from astrocytomas it is unlikely that the extracellular generation of H_2O_2 or $O_2^{\bullet-}$ are involved in these responses. However there may be other products of dopamine auto-oxidation which may elicit the GSH response. In support of this hypothesis the rise in intracellular GSH in mesencephalic cultures in response to L-dopa was attenuated by the addition of the reducing agent ascorbate (Mytilineou *et al*, 1993). In the current study inhibition of MAO with tranylcypromine or catechol-O-methyltransferase with Ro-41-0960 also had no effect on the GSH response to dopamine in neuroblastomas or astrocytomas. This indicates that increased flux through MAO which generates H_2O_2 as a side product was not involved in this response. Moreover this suggests that dopamine breakdown products including DOPAC, 3-methoxytryamine and HVA are unlikely to be involved in the alterations in GSH. SCH-23390, an antagonist of D_1 and D_5

receptors and sulpiride, an antagonist of D_2, D_3 and D_4 receptors also had no effect on the dopamine induced increase in GSH in neuroblastomas or increased release from astrocytomas. This indicates that this response was not mediated through classical dopamine receptor signalling. Comparable to dopamine the L-dopa induced increase in intracellular GSH in SH-SY5Y cells was also not inhibited by catalase, SOD, tranylcypromine, Ro-41-0960, SCH-23390 or sulpiride. Mytilineou *et al* (1993) also found that catalase, SOD and the MAO inhibitor pargyline had no effect on the L-dopa induced rise in intracellular GSH in mesencephalic cultures. However in the current study combined treatment of SH-SY5Y cells with L-dopa and catalase did lead to a small increase in intracellular GSH in comparison to cells treated with L-dopa alone. This increase could relate to lowered H_2O_2 due to the presence of extracellular catalase, in turn reducing intracellular oxidation of GSH by glutathione peroxidase.

The Nrf2-keap1 signalling pathway may potentially provide a mechanistic explanation not only for the increase in intracellular GSH seen in neuroblastoma cells but also for the increased release of GSH from astrocytoma cells. The molecular association between Nrf2 and Keap1 prevents Nrf2 from entering the nucleus and additionally increases degradation of Nrf2 via the ubiquitin-proteasome (Itoh *et al*, 1999; Zipper and Mulcahy, 2002; Itoh *et al*, 2003). Modification of keap1 at cysteine residues for example by ROS in combination with phosphorylation of Nrf2 leads to dissociation of Nrf2 from keap1 (reviewed by Kaspar *et al*, 2009). Nrf2 then translocates to the nucleus where it binds to AREs (antioxidant response elements), consensus sequences found upstream of multiple genes involved in antioxidant defence, and increases gene transcription (Rushmore *et al*, 1991; Venugopal *et al*, 1996; Itoh *et al*, 1997). Nrf2 in addition to up-regulating γ-glutamylcysteine ligase has also been demonstrated to induce expression of the multidrug resistance associated protein 1 (MRP1) a transporter protein involved in GSH release from astrocytes (Wild *et al*, 1999; Hayashi *et al*, 2003). The activation of this pathway can therefore increase both GSH synthesis and GSH release (Mulcahy and Gipp, 1995; Rahman et al, 1996; Hayashi *et al*, 2003; Minelli et al, 2009; reviewed by Peuchen *et al*, 1997). Furthermore it has recently been demonstrated that dopamine can activate AREs and that this activation was dependent on dopamine oxidation (Wang *et al*, 2010b). L-dopa was also found to activate AREs however this activation was much less potent than with dopamine, which perhaps could provide an explanation for the difference between the effects of L-dopa and dopamine

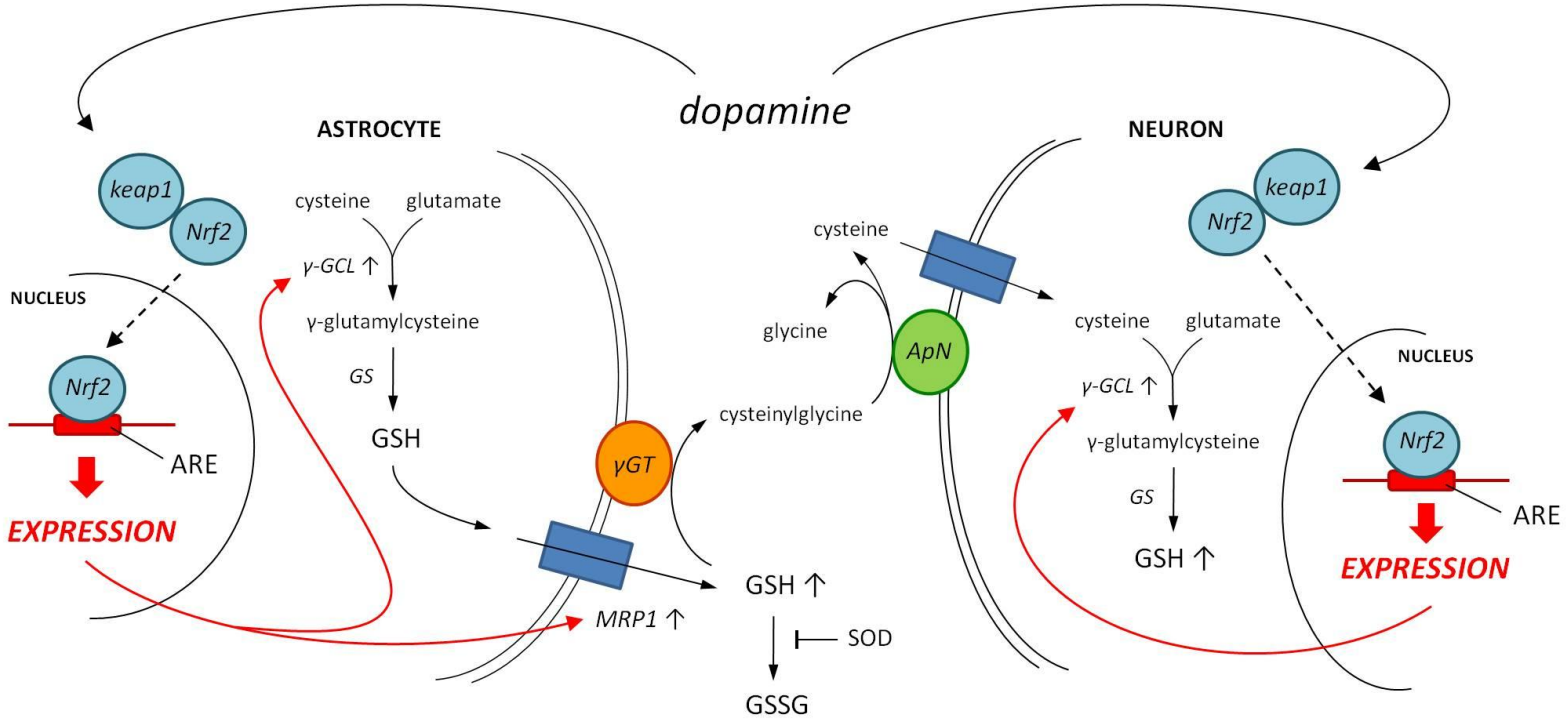


Figure 5.11 Proposed mechanism for the dopamine induced increase in GSH within neuronal-like cells and GSH release from astrocyte-like cells. γ-GCL: γ-glutamylcysteine ligase, GS: glutathione synthase, GSH: reduced glutathione, MRP1: multidrug resistance protein 1, γ-GT: γ-glutamyltranspeptidase, ApN: aminopeptidase N, Nrf2: nuclear factor erythroid 2-related factor, keap1: kelch-like ECH associating protein 1, ARE: antioxidant responsive element, SOD: superoxide dismutase, GSSG: glutathione disulphide.

in the current study. It will be of importance to further investigate whether the activation of AREs is involved in the observed GSH responses in neuroblastomas and astrocytomas. Furthermore investigation of the control mechanism by which the two cell types respond differentially to the same signal could be significant to an understanding of how the central nervous system responds to oxidative stress.

In the current set of experiments L-dopa was unable to induce an increase in GSH in neuroblastoma cells unless it was first converted to dopamine by AADC. Similarly astrocytoma cells were unresponsive to L-dopa and do not express AADC activity. This suggests that dopamine or a product of dopamine auto-oxidation could be a specific signal for up-regulation of GSH within the brain. Accordingly in AADC deficiency, where there is a severe deficit of dopamine, this signal may be absent. In consequence the brains of AADC deficient patients may have decreased levels of GSH or decreased control over GSH. The result of this could be decreased antioxidant defences and therefore an increased vulnerability to ROS. Further work will be required in both primary neuronal cultures and *in vivo* in order to further understand the physiological and pathophysiological relevance of these observations. Measuring GSH in the CSF of patients with AADC deficiency could be a potentially important experiment to ascertain whether GSH is decreased and therefore whether antioxidant therapy could be of benefit for these patients.

5.6.4 Complex I and dopamine

GSH redox cycling forms one of the major antioxidant defence systems within the mitochondrial matrix (Jocelyn and Dickson, 1980; Martensson *et al*, 1990; Heales *et al*, 1995). Furthermore activity of the respiratory chain is sensitive to changes in GSH status (Heales *et al*, 1995; Bolanos *et al*, 1996; Merad-Boudia *et al*, 1998; Merad-Saidoune *et al*, 1999; Chinta and Andersen, 2006). Alterations in GSH levels such as observed within SH-SY5Y cells suggested that there may be other mitochondrial changes and consequently the activity of the mitochondrial respiratory chain was investigated. The activity of complex I of the mitochondrial respiratory chain was increased in response to dopamine treatment in SH-SY5Y cells. However previous studies have found that dopamine negatively effects respiration (Ben Shachar *et al*, 1995; Cohen *et al*, 1997; Berman and Hastings; 1999; Gluck *et al*, 2002; Gluck and Zeevalk, 2004; Brenner-Lavie *et al*, 2008; Brenner-Lavie *et al*, 2009). *In vitro* 10µM dopamine treatment has been demonstrated to act as an inhibitor of complex I activity

possibly via dopamine auto-oxidation (Ben Shachar *et al*, 1995). In isolated intact mitochondria dopamine has also been shown to reversibly reduce respiration, measured as O_2 consumption (Cohen *et al*, 1997; Berman and Hastings; 1999; Gluck *et al*, 2002; Gluck and Zeevalk, 2004). This effect was found to be dependent on MAO and H_2O_2 generation indicating that dopamine degradation via MAO producing H_2O_2 as a side product was responsible for this effect (Cohen *et al*, 1997; Berman and Hastings; 1999). Additionally in isolated mitochondria dopamine-quinone, a product of dopamine auto-oxidation, was found to lead to uncoupling of electron transport from ATP synthesis, possibly indicating an increase in proton leakage across the mitochondrial inner membrane (Berman and Hastings, 1999). Treatment of SH-SY5Y cells with 100µM dopamine was shown to depolarise the mitochondrial membrane potential by ~30%, which may have been related to inhibition of complex I (Brenner-Lavie *et al*, 2008). Furthermore dopamine treatment of SH-SY5Y cells over 10min led to a 20% reduction in respiration measured as O_2 consumption (Brenner-Lavie *et al*, 2009). However this decrease was not sustained in 24hr and 48hr treatments, unless cells were treated concomitantly with tranylcypromine. The above studies indicate that dopamine either directly or via ROS production can decrease mitochondrial respiration possibly through an effect on complex I. However none of these studies directly measured complex I activity in cells following treatment with dopamine.

The increase in complex I activity after dopamine treatment in the current study could suggest a compensatory up-regulation as a result of decreased respiration or increased ROS following dopamine treatment. In support of this L-dopa administration to rats was found to lead to an increase in mitochondrial complex I activity and protein in the substantia nigra and was associated with increased dopamine concentrations (Calabrese *et al*, 2007). This up-regulation was related to an induction of heat shock proteins which are potentially involved in the cellular response to oxidative stress (Omar and Pappolla, 1993; Calabrese *et al*, 2003). Alternatively the increase in intracellular GSH observed in response to dopamine treatment in the present study could be responsible for the increase in complex I activity. The activity of complex I has been demonstrated to be sensitive to GSH concentrations such that decreased GSH increases the vulnerability of complex I to oxidative damage (Heales *et al*, 1995; Bolanos *et al*, 1996; Merad-Boudia *et al*, 1998; Merad-Saidoune *et al*, 1999; Chinta and Andersen, 2006). Potentially the heightened level of GSH may surmount a basal level of ROS mediated damage allowing complex I to function with a higher activity. Conceivably if dopamine

increases complex I activity at physiological levels then a consequence of dopamine deficiency, such as in AADC deficiency, would be a reduction in complex I activity. Notably L-dopa did not have the same effect on complex I indicating that this is a specific effect of dopamine. In isolated Guinea pig liver mitochondria approximately 50-60% of ubiqunione exists as reduced ubiquinol during respiration (Takada *et al*, 1984). Increased complex I activity in response to dopamine may increase the proportion of ubiquinol in the mitochondrial inner membrane. In addition to its role in oxidative phosphorylation ubiquinol can also act as an antioxidant and may be particularly effective at terminating lipid peroxidation (Frei *et al*, 1990; reviewed by James *et al*, 2004). Conversely the ubisemiquinone radical formed from one electron oxidation of ubiquinol can react with O_2 to produce $O_2^{\bullet-}$ and subsequently H_2O_2 which potentially may propagate further ROS generation (Frei *et al*, 1990; Schultz *et al*, 1996). Consequently increased complex I activity could have both antioxidant and pro-oxidant effects. However further work is required in an *in vivo* model of AADC deficiency to ascertain if there are alterations of mitochondrial function in the brains of AADC deficient patients and to ascertain the pathophysiological relevance of this observation.

5.7 Conclusions

The state of AADC deficiency leads to an accumulation of L-dopa and a deficiency of dopamine. Evidence presented in this chapter indicates that L-dopa accumulation may potentially lead to damage to dopaminergic neurons. Furthermore dopamine has been shown to potentially increase GSH concentrations in the brain by increasing GSH levels in neuronal-like cells and GSH release from astrocyte-like cells. Consequently the dopamine deficit in AADC deficiency could lead to an impairment of antioxidant responses within the brain. This could provide an indication that the catecholaminergic neurons of AADC deficient patients could have a heightened susceptibility to oxidative stress that could possibly lead to cell death. Additionally as dopamine treatment increased complex I activity in neuroblastoma cells it is also possible that dopamine deficiency may lead to a reduction in complex I in catecholaminergic neurons. However further work will be required in an animal model of AADC deficiency, in patient samples and in post mortem tissue to ascertain the relevance of these observations for patients with AADC deficiency.

Chapter 6

The influence of pyridoxal 5'-phosphate availability on aromatic L-amino acid decarboxylase

6.1 Introduction

Pyridoxal 5'-phosphate (PLP) the active form of vitamin B_6 acts as a coenzyme for the decarboxylation of aromatic amino acids by AADC. Vitamin B_6 usually in the form of pyridoxine is a common although not universal treatment of AADC deficiency (Brun *et al*, 2010). This treatment was administered in the first two AADC deficiency cases in an attempt to bolster residual AADC enzyme activity (Hyland *et al*, 1992). In response to this treatment CSF levels of L-dopa and 3-O-methyldopa were reduced and homovanillic acid concentrations were increased, suggestive of an improvement in dopamine metabolism. In another patient vitamin B_6 treatment was associated with increased plasma serotonin and decreased 3-O-methyldopa and L-dopa (Maller *et al*, 1997). However in this case there was no concomitant improvement in clinical symptoms. Furthermore vitamin B_6 treatment in the majority of reported cases has not led to any substantial improvement in clinical picture (Swoboda *et al*, 1999; Pons *et al*, 2004).

The majority of ingested pyridoxine is converted to PLP in the liver by sequential phosphorylation and oxidation steps catalysed by pyridoxal kinase and pyridox(am)ine 5'-phosphate oxidase (PNPO), respectively (McCormick and Snell, 1959; McCormick *et al*, 1961; Wada and Snell, 1961). PLP is then released into circulation but must be dephosphorylated by tissue non-specific alkaline phosphatase prior to crossing the blood-brain barrier and/or being transported into cells (Lumeng *et al*, 1974; Lumeng *et al*, 1980; Whyte *et al*, 1985). Within cells pyridoxal is re-phosphorylated by pyridoxal kinase to produce the active coenzyme. Intracellular AADC is then able to bind PLP and consequently exert its catalytic activity. However, PLP also acts as a coenzyme for a wide array of enzymatic reactions and can interact with a range of other proteins including hormone receptors and transcription factors (Allgood *et al*, 1990; Allgood *et al*, 1993; Huq *et al*, 2007). The relative complexity of PLP metabolism and pharmacokinetics, and the multitude of potential sites of action perhaps suggest that the efficacy of vitamin B_6 treatment in AADC deficiency may depend on multiple factors.

One potential efficacious effect of vitamin B_6 treatment could be to influence the availability of active AADC. It has previously been demonstrated that PLP can act to stabilise some enzymes and prevent their degradation. For example the activity of the PLP-dependent enzyme tyrosine aminotransferase (TAT) in rat liver has been shown to

increase in a dose-dependent and protein synthesis dependent manner in response to vitamin B_6 treatment (Greengard and Gordon, 1963). In addition it has been demonstrated that the half-life of TAT is increased in rat liver after pyridoxine administration (Snape *et al*, 1980). Later PLP was found to protect TAT from degradation by the ubiquitin-proteasome *in vitro* (Gross-Mesilaty *et al*, 1997). Taken together these findings indicate that PLP may act as a chaperone, preventing the degradation of newly synthesised TAT via the proteasome. PLP is also known to influence expression of other proteins through interaction with transcription factors. Treatment with a vitamin B_6 antagonist or pyridoxine respectively increased or reduced glucocorticoid receptor mediated gene expression through a mechanism involving the transcription factor nuclear factor 1 (Allgood *et al*, 1990; Allgood *et al*, 1993). Furthermore PLP has been found to bind to a specific lysine residue of the nuclear receptor interacting protein 140 (RIP140; Huq *et al*, 2007). This interaction of PLP with RIP140 prevented RIP140 export from the nucleus and increased its transcriptional repressor activity.

Vitamin B_6 deficiency in rodents has been found to reduce AADC activity in a range of tissues including brain (Rahman *et al*, 1982; Siow and Dakshinamurti, 1985; Guilarte *et al*, 1987). In each study AADC activity was measured both with and without the addition of PLP to the reaction mixture. Adding PLP to the reaction did not return AADC activity to control levels indicating that the amount of active AADC may have been reduced. In rat pheochromocytoma cells cultured in vitamin B_6 deficient medium together with 4-deoxypyridoxine, a vitamin B_6 antagonist, both AADC activity and AADC protein were reduced (Matsuda *et al*, 2004). Together these findings indicate that as well as acting as a co-enzyme PLP could play an additional role in maintaining the availability of active AADC. In addition to AADC deficiency vitamin B_6 has been suggested to be a beneficial treatment for drug-induced Parkinsonism, tardive dyskinesia and some Parkinson's disease patients treated with L-dopa and a peripheral decarboxylase inhibitor (Sandyk and Pardeshi, 1990; Tan *et al*, 2005; Lerner *et al*, 2007). The benefits of vitamin B_6 treatment in these conditions could relate to increased AADC activity, due to increased availability of PLP, in turn leading to improvements in monoamine metabolism. An improved understanding of the effect of PLP on AADC availability may potentially explain the efficacy of vitamin B_6 treatment in these conditions and could indicate previously unknown benefits of vitamin B_6 treatment for AADC deficiency.

6.2 Aims

1. To determine the effect of altered PLP availability on AADC in patients with PNPO deficiency and in cell culture

2. To determine the mechanism by which PLP influences AADC availability

6.3 Acknowledgement

Pat of this work has been published (Allen *et al*, 2010). All experimental work was my own with the exception of PLP measurement, which was performed by Viruna Neergheen and Marcus Oppenheim (Neurometabolic Unit, National Hospital) and measurement of the AADC kinetics of one patient with PNPO deficiency which was undertaken by Keith Hyland (Horizon Molecular Medicine LLC, Atlanta, GA, USA).

6.4 Methods

6.4.1 Patient Samples

Patient samples were lithium heparin plasma. Plasma AADC activity was measured in 5 control patients (3 male, 2 female; age range: 2 months to 3 years), 6 patients with AADC deficiency (3 male, 3 female; age range: 7 months to 6 years) and two patients with PNPO deficiency (2 male; age range: 2 weeks to 6 weeks). All patient samples were tested as part of diagnostic investigations requested by the patient's clinician and after informed consent. Anonymised control samples were from patients found not to have conditions relating to vitamin B_6 or monoamine metabolism used in accordance with accredited Neurometabolic Unit (National Hospital for Neurology and Neurosurgery) procedures.

6.4.2 Treatment solutions

200mM 4-deoxypyridoxine, 10mM PLP and 10mM pyridoxal were made up as stock solutions in HPLC grade H_2O, 0.2µM sterile filtered and stored at -20˚C. The PLP stock solution was protected from light. 3mg/mL cycloheximide was made up in HPLC grade H_2O, 0.2µM sterile filtered and stored at +4˚C.

6.4.3 Cell culture

Routine cell culture of SH-SY5Y cells was performed as described in section 2.2.6.

6.4.4 AADC Activity

AADC activity with either L-dopa or 5-HTP as substrate was measured as described in section 3.3.3 and section 3.3.4.

6.4.5 Measurement of PLP

Cells were prepared as described in section 3.3.2 and PLP was measured as described in section 2.2.5.

6.4.6 Measurement of catecholamines

Cells and medium were prepared as described in section 2.2.8.1 and catecholamines measured as described in section 2.2.1.

6.4.7 Western Blotting

Cells were prepared as described in section 2.2.8.4 Western blotting was performed as described in section 2.5.

6.4.8 Quantitative PCR

RNA was isolated as decribed in section 2.6.2. RT-PCR was performed as described in section 2.6.3 and quantitative PCR was performed as described in section 2.6.5

6.5 Experimental Protocol

6.5.1 AADC activity and PLP measurement

SH-SY5Y cells in DMEM/F-12 + 100mL/L FBS were counted using a Neubauer improved hemocytometer and the appropriate quantity was centrifuged at 500xg for 5min at room temperature. Cells were then resuspended either in DMEM/F-12 + 100mL/L FBS or in pyridoxine deficient DMEM/F-12 + 100mL/L FBS. Cells were seeded at a density of 5.00×10^4 cells/cm^2 in 6-well cell culture dishes, the surface area of each well was $9.6cm^2$. 75µL 200mM 4-deoxypyridoxine (final conc. 5mM), 75µL PLP (400µM: final conc. 10µM or 4mM: final conc. 100µM) or 75µL 4mM pyridoxal (final conc. 100µM) was added to appropriate treated wells. 75µL sterile HPLC grade H_2O was added to untreated wells. Plates were incubated at +37˚C in 5.0% CO_2 for 72hr. For determination of AADC activity cells were processed as indicated in section 6.4.4 and for PLP determination as indicated in section 6.4.5.

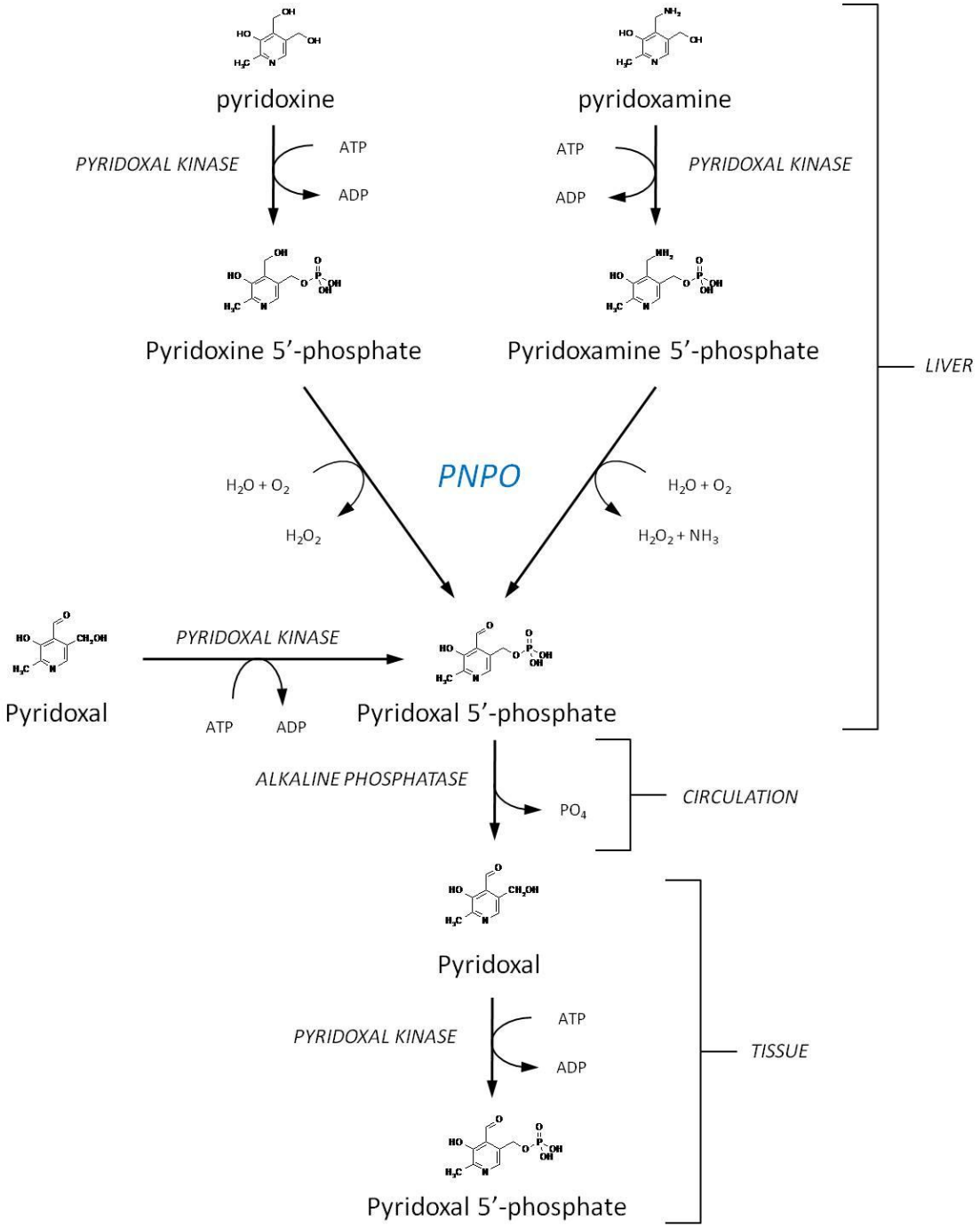


Figure 6.1 Pyridoxal 5'-phosphate metabolic pathway in humans. ATP: adenosine triphosphate, ADP: adenosine diphosphate. PNPO: pyridoxamine 5'-phosphate oxidase.

6.5.2 AADC enzyme kinetics, mRNA expression and western blotting

SH-SY5Y cells in DMEM/F-12 + 100mL/L FBS were counted using a Neubauer improved hemocytometer and the appropriate quantity was centrifuged at 500xg for 5min at room temperature. Cells were then resuspended either in DMEM/F-12 + 100mL/L FBS or in pyridoxine deficient DMEM/F-12 + 100mL/L FBS. Cells were seeded at a density of 5.00 x 10^4 cells/cm^2 in 56.7cm^2 cell culture dishes. 312.5µL 200mM 4-deoxypyridoxine (final conc. 5mM) or 312.5µL 400µM PLP (final conc. 10µM) were added to appropriate dishes. 312.5µL sterile HPLC grade H_2O was added to untreated dishes. Plates were incubated at +37˚C in 5.0% CO_2 for 24hr or 72hr. For determination of AADC enzyme kinetics cells were processed as indicated for AADC activity in section 6.4.4, for mRNA analysis cells were processed as indicated in section 6.4.8 and for western blotting cells were processed as indicated in section 6.4.7.

6.5.3 AADC stability

The stability of AADC was determined by a previously described method (Moore *et al*, 2003), where AADC activity was monitored in the absence of protein synthesis to determine whether the degradation rate had increased in response to treatment. Cycloheximide was added at a concentration previously used to inhibit protein synthesis (Iredale *et al*, 1996; Kalfon *et al*, 2006). Cells were cultured and treated as described in section 6.5.1. After the 72hr incubation 100µL 3mg/mL cycloheximide was added to each well. Cells were either harvested immediately or incubated for 4hr or 8hr at 37˚C in 5.0% CO_2. Cells were harvested and processed as indicated for AADC activity (see section 6.4.4).

6.5.4 Catecholamines measurement

For catecholamine measurement cells were cultured and treated as described in section 6.5.1. After the 72hr incubation 62.5µL 10mM L-dopa (final conc. 50µM) was added to all dishes and incubated for 1hr at +37˚C in 5.0% CO_2. Cells and cell culture medium were then processed as indicated for catecholamine measurement (see section 6.4.6).

6.6 Results

6.6.1 Plasma AADC activity in PNPO deficient patients

For all AADC assays, with either L-dopa or 5-HTP as substrate using patient or cell culture samples, PLP at a saturating level of 70µM was incubated at +37°C for 120 minutes prior to the addition of substrate. Therefore it is unlikely that AADC activity was dependent on the concentration of PLP coenzyme present during the assay. Plasma AADC activity with L-dopa as substrate was determined in two patients with PNPO deficiency (see section 1.4.4). L-dopa decarboxylation was below the paediatric reference range in both cases (reference range: 36-129 pmol/min/mL) and additionally was reduced in comparison to controls of a similar age (see figure 6.2). Plasma AADC activity was also determined in six paediatric patients with AADC deficiency. The activity of the majority of these patients was almost undetectable and in all cases below that observed for the PNPO deficient patients (see figure 6.2). The enzyme kinetics of AADC for PLP was also determined for one patient with PNPO deficiency by measuring L-dopa decarboxylation in plasma at different concentrations of PLP (0.5 to 70.0µM). The coenzyme K_m apparent of this patient was found to be increased to 15.0µM in comparison to control (3.1 ± 0.4µM; see section 3.4.3; the K_m apparent for the PNPO patient was measured by Keith Hyland (Horizon Molecular Medicine LLC, Atlanta, GA, USA).

6.6.2 AADC activity in SH-SY5Y cells

SH-SY5Y cells were exposed to four different treatments aimed at altering PLP availability or metabolism. In order to increase PLP availability cells were cultured with 10µM PLP in DMEM/F-12, which contains 9.71µM pyridoxine (Invitrogen). Cells were also cultured in pyridoxine deficient (-B_6) medium in order to deplete cells of PLP. Additionally cells were cultured with the vitamin B_6 antagonist 4-deoxypyridoxine (4-DP) at a 5mM concentration in either -B_6 culture medium or control culture medium. AADC activity with L-dopa as substrate was measured in cells exposed to the different vitamin B_6 conditions for 72hr. Cells cultured in -B_6 medium were found to have significantly reduced L-dopa decarboxylation activity to

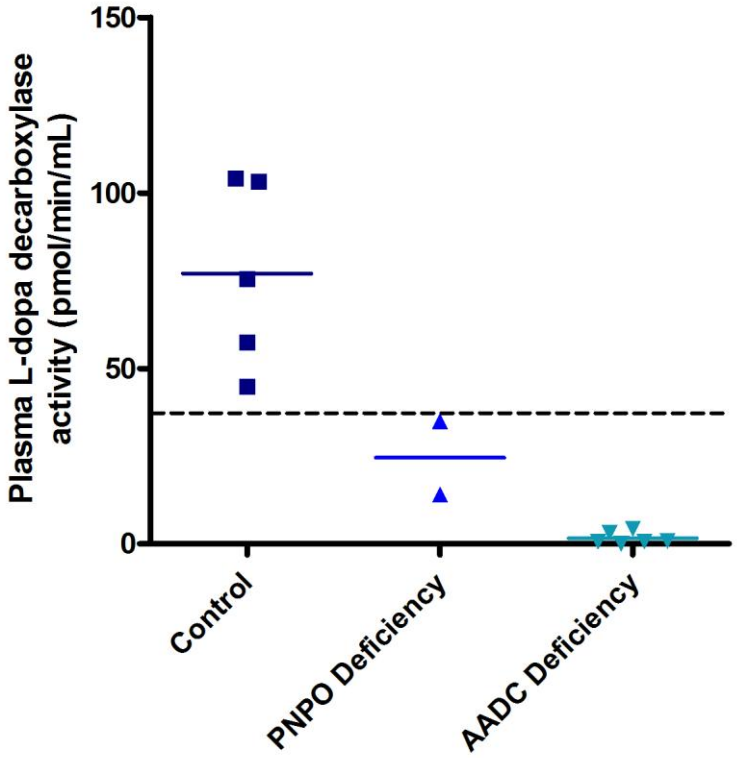


Figure 6.2 Plasma AADC L-dopa decarboxylase activity in paediatric disease control, PNPO deficient and AADC deficient patients. ------ Indicates lower limit of paediatric reference range (36 to 129 pmol/min/mL).

approximately 70% of control ($p < 0.05$; see figure 6.3a). 4-DP treatment of cells cultured in -B_6 medium led to further decreases in L-dopa decarboxylation to approximately 30% of control ($p < 0.001$). L-dopa decarboxylation was decreased to a similar extent when cells cultured in control medium were treated with 4-DP. L-dopa decarboxylation was not significantly different between 4-DP treated cells in control or -B_6 medium ($p = 0.526$; see figure 6.3a). Due to the similarity between the two 4-DP conditions only 4-DP treated cells grown in -B_6 medium were investigated in further experiments. This treatment was chosen to allow a direct comparison to be made to the work of Matsuda *et al* (2004) who also used this treatment.

AADC activity with 5-HTP as substrate was altered to a similar extent to that of L-dopa decarboxylation following the same treatments. SH-SY5Y cells cultured for 72hr in -B_6 medium had reduced AADC activity with 5-HTP to approximately 70% of control ($p < 0.001$) and activity was further reduced to approximately 30% of control with 4-DP

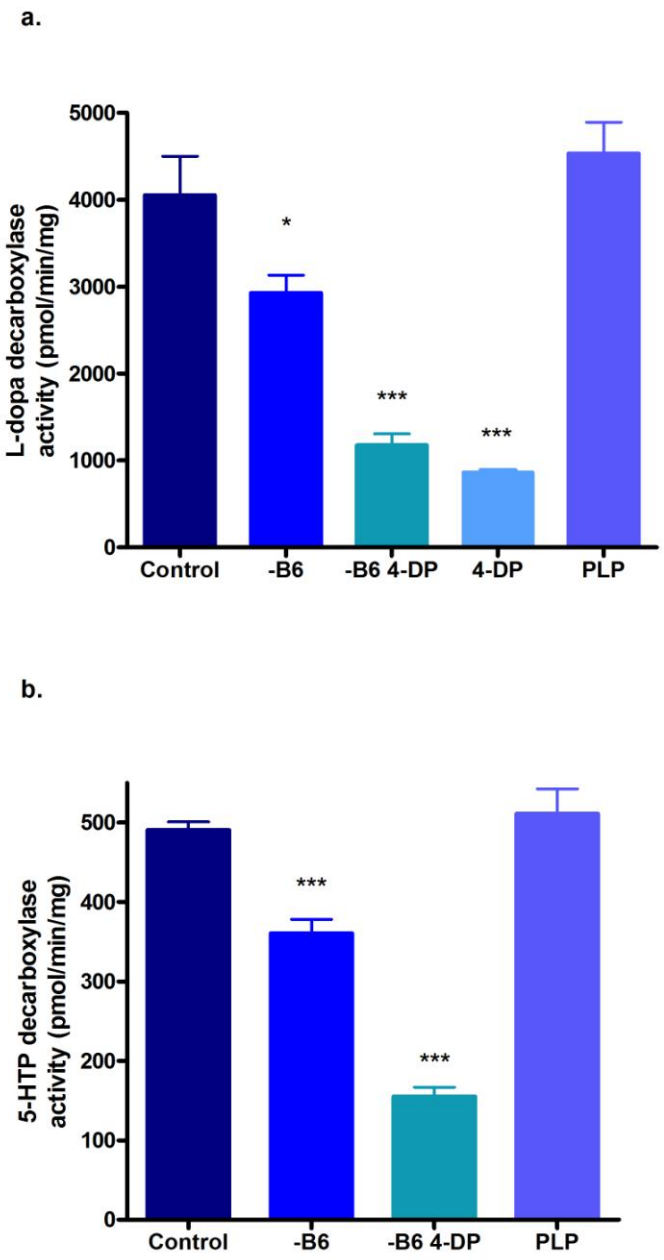


Figure 6.3 AADC activity in SH-SY5Y homogenates following vitamin B_6 treatments. AADC activity with a. L-dopa and b. 5-HTP as substrate. Cells were cultured for 72hr under the following conditions: -B6, vitamin B6 deficient medium; -B6 4-DP, vitamin B6 deficient medium + 5mM 4-deoxypyrdioxine; 4-DP: control medium + 5mM 4-deoxypyridoxine; PLP, control medium + 10µM PLP. Results are mean ± SEM of 5-11 independent experiments. *p <0.05, ***p <0.001, difference in comparison to control by ANOVA followed by least significant difference test.

treatment coupled with -B_6 medium ($p < 0.001$; see figure 6.3b). In cells treated with 10µM PLP there was no statistically significant change in AADC activity with either L-dopa or 5-HTP as substrate compared to control ($p = 0.283$; $p = 0.482$, respectively). To ascertain whether 4-DP had a direct inhibitory effect on AADC it was added *in vitro* to SH-SY5Y homogenates at the same time point as PLP during the AADC assay with L-dopa as substrate. 4-DP was not found to have any direct effect on AADC activity (see figure 6.4)

6.6.3 PLP in SH-SY5Y cells

PLP was measured by Viruna Neergheen and Marcus Oppenheim (Neurometabolic Unit, National Hospital). The intracellular levels of PLP were determined after the cells had been treated for 72hr with the different vitamin B_6 conditions. 72hr culturing in -B_6 medium or in -B_6 medium with 4-DP led to significant reductions in intracellular PLP concentrations to 66% and 67% of control respectively ($p < 0.01$ and $p < 0.05$, respectively; see figure 6.5). This indicates that 4-DP treatment does not directly affect the intracellular availability of PLP. Treatment of SH-SY5Y cells with 10µM PLP for 72hr did not increase intracellular PLP concentrations. Cells were also treated with 100µM PLP which also did not lead to increases in intracellular PLP at the 72hr time point (see figure 6.5). As PLP must be dephosphorylated prior to cellular uptake the ability of pyridoxal to increase intracellular PLP concentrations was also investigated. Treatment with 100µM pyridoxal also did not increase intracellular PLP concentrations after 72hr (see figure 6.5).

6.6.4 Substrate Kinetics

The coenzyme K_m of AADC for PLP could not be determined in SH-SY5Y cell culture samples due to the high proportion of AADC that was already bound to PLP (see section 3.5.3). Consequently the altered coenzyme K_m found in the PNPO deficient patient could not be further investigated in this cell culture model. In order to characterise the alterations found in AADC activity in cells exposed to the different vitamin B_6 treatments the substrate kinetics of AADC was determined in culture extracts following 72hr of treatment. Substrate kinetics were determined by varying the concentration of L-dopa (25 to 2000µM) or 5-HTP (5 to 500µM) present during the reaction. L-dopa decarboxylation appeared to follow classical Michaelis-Menten kinetics in samples from all four conditions with the reaction saturated by 1000µM of

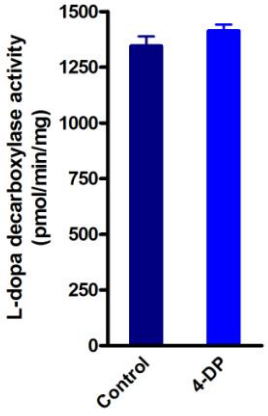


Figure 6.4 AADC activity with L-dopa as substrate in SH-SY5Y cell homogenates in the presence of 4-deoxypyridoxine. 4-DP: 5mM 4-deoxypyridoxine (was added to homogenate at the start of the assay, the final concentration of 4-deoxypyridoxine during the assay was 500µM. Results are mean ± SEM of 5 to 11 independent experiments

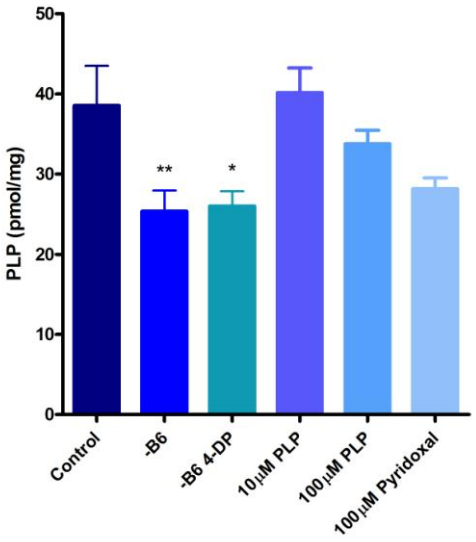


Figure 6.5 Intracellular concentration of PLP in SH-SY5Y cells after vitamin B_6 treatments. Cells treated for 72hr with -B6, vitamin B_6 deficient medium; -B6 4-DP, vitamin B_6 deficient medium + 5mM 4-deoxypyrdioxine; or with PLP or pyridoxine at the indicated concentration. Results are mean ± SEM of 3-5 independent experiments. *p <0.05, **p <0.01, difference in comparison to control by ANOVA followed by least significant difference test.

L-dopa in each treatment. However the rate of reaction achieved by the point of saturation was reduced compared to control in cells cultured in -B_6 medium and further reduced in 4-DP treated cells also cultured in -B_6 medium (see figure 6.6a). The same was found with 5-HTP as substrate where the reaction for each vitamin B_6 condition was saturated by 250µM of 5-HTP. The rate of reaction achieved at saturation was lower in cells cultured in -B_6 medium and lower still in cells treated with 4-DP and cultured in -B_6 medium (see figure 6.6b). In cells treated with 10µM PLP small increases in the reaction rate at saturation were found with either 5-HTP or L-dopa as substrate. V_{max} and substrate K_m for each condition were determined by Lineweaver-Burk plots (see figure 6.7a and 6.7b). The K_m apparent for the substrate with either L-dopa or 5-HTP was not significantly different for any of the vitamin B_6 conditions in comparison to control (see table 6.1). Note that this is the substrate K_m that was unchanged in this experiment not the coenzyme K_m, which was found to be altered in one PNPO deficient patient. However the V_{max} with L-dopa as substrate was decreased by approximately 30% ($p < 0.05$) and 75% ($p < 0.001$) in cells grown in -B_6 medium and in 4-DP treated cells grown in -B_6 medium respectively (see table 6.1). With 5-HTP as substrate V_{max} was reduced compared to control by approximately 36% ($p < 0.05$) and 74% ($p < 0.001$) in cells grown in -B_6 medium and in 4-DP treated cells grown in -B_6 medium respectively. Cells treated with 10µM PLP showed no statistically significant change in V_{max}.

6.6.5 AADC Protein, Stability and Expression

Western blotting was performed using an antibody raised against a C-terminal peptide of amino acids 464-475 of human AADC (Abcam). This antibody detected two bands at approximately 50kDa (see figure 6.8). These two bands are most likely two isoforms of AADC termed $AADC_{480}$ and $AADC_{442}$ which have predicted molecular masses of 54kDa and 50kDa respectively (O'Malley *et al*, 1995; Chang *et al*, 1996). $AADC_{442}$ is missing exon 3 and so consequently would still possess the recognition site for the antibody used in the current study. AADC protein was reduced in cells cultured in -B_6 medium as well as those cells also treated with 4-DP (see figure 6.8). Using quantification software (Bio-Rad), 4-DP treated cells cultured in -B_6 medium had the lowest level of AADC protein, 70 ± 5% of control (n = 3). AADC protein was reduced to a lesser extent in the cells cultured in -B_6 medium at 78 ± 13% of control (n = 4),

while control cells and cells treated with 10µM PLP had approximately equivalent levels of AADC protein.

The stability of AADC was monitored by measuring the change in AADC activity with L-dopa as substrate after inhibition of protein synthesis with 100µg/mL cycloheximide. This protocol has been used previously in SH-SY5Y cells to investigate the stability of other proteins (Moore *et al*, 2003). Consistent with observations described above cells cultured for 72hr in -B_6 medium and those cultured in -B_6 medium with 4-DP had decreased levels of L-dopa decarboxylation, with greater reductions seen in 4-DP treated cells, in comparison to control (see figure 6.9a). Subsequent to this 72hr treatment AADC activity was measured at 4hr and 8hr after the addition of cycloheximide. No change in AADC activity with L-dopa was observed in control cells, cells cultured in -B_6 medium or 4-DP treated cells cultured in -B_6 medium across the 8hr incubation with cycloheximide (see figure 6.9a). This indicates that AADC stability is unaffected by these treatments over the tested time period. To establish whether the observed changes in the levels of AADC protein and activity in response to the vitamin B_6 treatments were related to changes in expression AADC mRNA levels were measured by quantitative PCR. AADC mRNA levels were normalised against GAPDH mRNA, GAPDH mRNA was not significantly different from control in any of the vitamin B_6 treatments tested at either 24hr or 72hr ($p = 0.206$; $p = 0.747$ respectively). There were no statistically significant changes in AADC mRNA expression across the treatment groups in comparison to control after 24hr. Likewise there were no statistically significant changes in AADC mRNA at 72hr in cells cultured in -B_6 medium or in cells treated with 10µM in comparison to control cells. However at 72hr of treatment AADC mRNA expression was significantly reduced in cells cultured in -B_6 medium treated with 4-DP in comparison to control cells ($p < 0.01$; see figure 6.9b).

6.6.6 Catecholamine metabolism following vitamin B_6 treatment

As AADC activity was reduced following treatment with -B_6 medium or -B_6 medium with 4-DP the effect of these treatments upon monoamine metabolism in intact SH-SY5Y cells was also investigated. AADC activity was found to be decreased by a similar level with either L-dopa or 5-HTP as substrate (see section 6.6.2) therefore only catecholamine metabolism was investigated as it was considered that this would also be

representative of indoleamine metabolism. Cells were cultured under the different vitamin B_6 treatment conditions for 72hr, after which a saturating concentration of L-dopa (50µM) was added for 1hr to allow a maximum level of catecholamine metabolism to be observed. Following this incubation cells were harvested and intracellular and extracellular levels of catecholamines determined. Intracellular and extracellular levels of dopamine and DOPAC were decreased and intracellular L-dopa increased in cells cultured in $-B_6$ medium with 4-DP in comparison to control (see table 6.2). Extracellular dopamine was also decreased in cells cultured in $-B_6$ medium alone compared to control, however intracellular dopamine and intra- and extracellular DOPAC were unaffected. HVA levels were not altered after any of the vitamin B_6 treatments. Treatment with 10µM PLP had no effect on L-dopa, dopamine or metabolites in comparison to control.

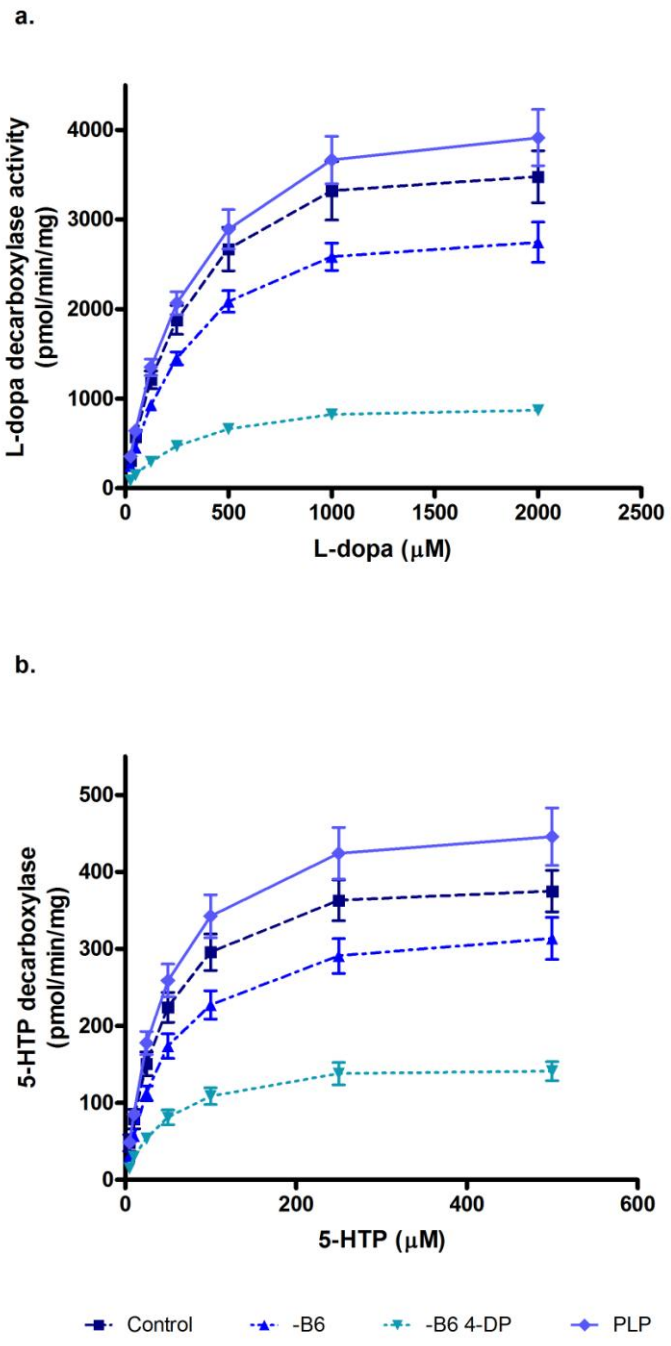


Figure 6.6 AADC enzyme kinetics in SH-SY5Y cell homogenates following vitamin B_6 treatments. Cells were treated for 72hr with -B6: vitamin B_6 deficient medium, -B6 4-DP: vitamin B_6 deficient medium + 5mM 4-deoxypyridoxine, control and PLP: 10µM PLP. a. L-dopa decarboxylase activity at varying concentrations of L-dopa (25 to 2000µmol/L). b. 5-HTP decarboxylase activity at varying concentrations of 5-HTP (5 to 500µmol/L). Results are mean ± SEM of 5-6 independent experiments.

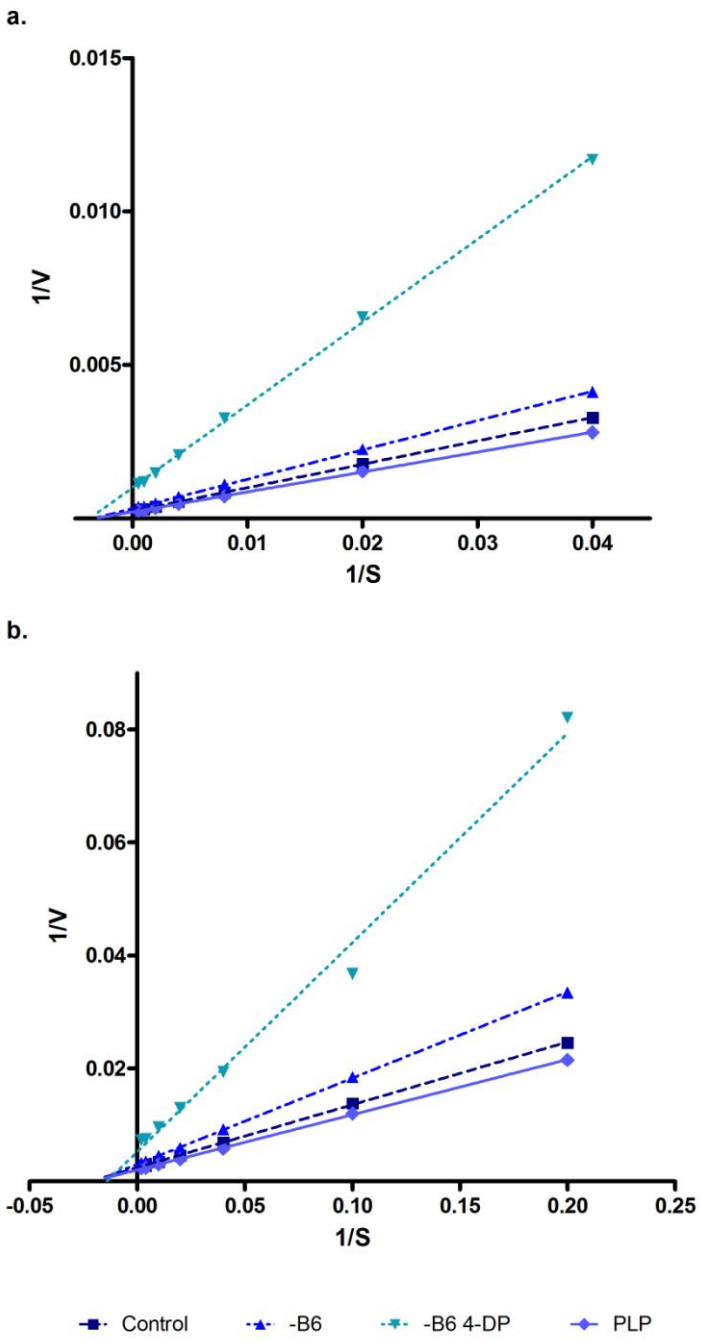


Figure 6.7 Lineweaver-Burk double reciprocal plot of SH-SY5Y cell homogenates following vitamin B_6 treatments. a. 1/S [1/L-dopa (μM)] against 1/V [1/L-dopa decarboxylase activity (pmol/min/mg)] b. 1/S [1/5-HTP (μM)] against 1/V [1/5-HTP decarboxylase activity (pmol/min/mg)].). Results are mean ± SEM of 5-6 independent experiments.

	Control	-B6	-B6 4-DP	PLP
L-dopa				
K_m	333 ± 62	307 ± 44	284 ± 57	302 ± 31
V_{max}	4453 ± 588	3199 ± 264*	1071 ± 150***	4705 ± 309
5-HTP				
K_m	47 ± 5	53 ± 6	45 ± 8	50 ± 2
V_{max}	426 ± 23	314 ± 10*	152 ± 11***	515 ± 46

Table 6.1 K_m and V_{max} of SH-SY5Y cell homogenate following vitamin B_6 treatments. K_m (μM) and V_{max} (pmol/min/mg) values for AADC with L-dopa and 5-HTP as substrates. SH-SY5Y cells were cultured for 72hr under the following conditions. -B6: vitamin B_6 deficient medium, -B6 4-DP: vitamin B_6 deficient medium + 5mM 4-deoxypyridoxine, control and PLP: 10μM PLP. Results are mean ± SEM of 5-6 independent experiments. * $p < 0.05$, *** $p < 0.001$, difference in comparison to control by ANOVA followed by least significant difference test.

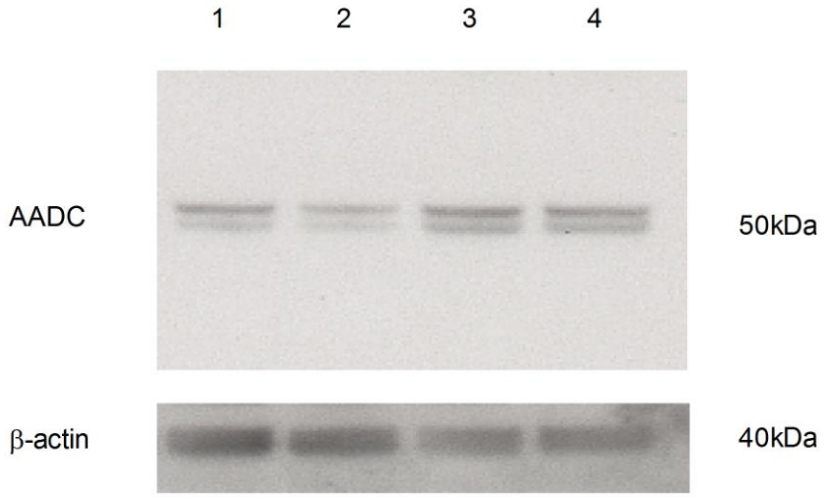


Figure 6.8 AADC protein in SH-SY5Y cells after vitamin B_6 treatments. Western blot of SH-SY5Y cell homogenates probed for AADC and re-probed for β-actin as a loading control. Cell treatment in lanes: 1. vitamin B_6 deficient medium, 2. vitamin B_6 deficient medium + 5mM 4-deoxypyridoxine, 3. control, 4. 10μM pyridoxal 5'-phosphate. Representative blot of 3-4 independent experiments.

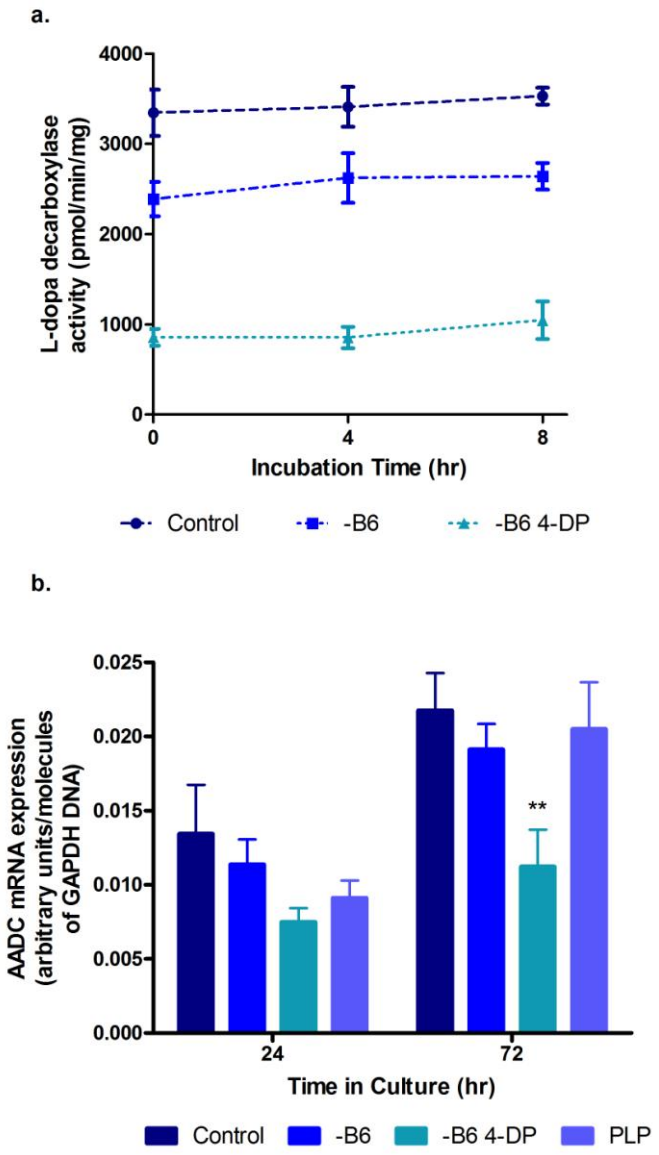


Figure 6.9 AADC stability and mRNA after vitamin B_6 treatments. a. AADC stability. 100µg/mL cycloheximide was added after 72hr of vitamin B_6 treatment and L-dopa decarboxylation was measured at 0, 4 and 8hr. Results are mean ± SEM of 5-6 independent experiments. b. AADC mRNA expression levels measured by quantitative PCR expressed as arbitrary units/ molecules of GAPDH DNA. Results are mean ± SEM of 3-5 independent experiments. Treatments: -B6: vitamin B_6 deficient medium, -B6 4-DP: vitamin B_6 deficient medium + 5mM 4-deoxypyridoxine, control and PLP: 10µM PLP. ** $p < 0.01$, difference in comparison to control by ANOVA followed by least significant difference test.

Treatment	L-dopa	dopamine	DOPAC	HVA
INTRACELLULAR				
Control	ND	1475 ± 666	823 ± 151	41 ± 4
-B6	ND	1294 ± 578	730 ± 72	28 ± 10
10µM PLP	1 ± 1	1789 ± 165	628 ± 74	34 ± 6
-B6 + 4-DP	28 ± 13*	933 ± 157*	165 ± 53**	27 ± 12
EXTRACELLULAR				
Control	NM	913.04 ± 130	216.69 ± 56	ND
-B6	NM	357.01 ± 144*	168.8 ± 89	ND
10µM PLP	NM	530.8 ± 196	187.68 ± 40	ND
-B6 + 4-DP	NM	125.07 ± 63**	ND	ND

Table 6.2 Catecholamine levels in SH-SY5Y cells following vitamin B_6 treatments. Cells treated for 72hr before the addition of 50µM L-dopa for 1hr prior to harvesting. Treatment conditions: -B6: vitamin B_6 deficient medium, -B6 4-DP: vitamin B_6 deficient medium + 5mM 4-deoxypyridoxine, control and PLP: 10µM PLP. NM: not measured, ND: not detectable. Results are mean ± SEM of 3 independent experiments. *p <0.05 , ** p <0.01, difference in comparison to control by ANOVA followed by least significant difference test.

6.7 Discussion

6.7.1 AADC availability is reduced by PLP deficiency

The activity of AADC was decreased in two patients with PNPO deficiency and in cultured neuroblastoma cells with reduced levels of PLP. In cell culture the reduction in activity was associated with a decreased V_{max} but not to changes in substrate K_m. This is suggestive of a reduction in active enzyme rather than a change in the interaction between enzyme and substrate, as would be indicated by a change in K_m. Furthermore in cells with reduced levels of PLP there was also a loss of AADC protein. Taken together this evidence suggests that deficiency of PLP causes a reduction in active AADC protein. PLP has previously been demonstrated to participate in mechanistic actions beyond its coenzyme function. For example PLP can act to prevent the degradation of the PLP-dependent enzyme TAT (Greengard and Gordon, 1963; Snape *et al*, 1980; Gross-Mesilaty *et al*, 1997). However in the current study there was no observed change in AADC stability for up to 8 hr in response to PLP deficiency. Consequently a similar mechanism to that observed for TAT is unlikely to account for the observed changes in AADC. The observed increase in the K_m apparent of AADC for PLP in one PNPO deficient patient may perhaps indicate a reduced efficiency of the interaction between AADC and PLP. This could indicate that PLP has a role in AADC folding or dimerisation and so consequently PLP deficiency could lead to an abundance of less mature forms of AADC which may have an altered K_m for PLP. Alternatively PNPO deficient patients are likely to accumulate PLP precursors, pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate, which at high concentrations may possibly compete with PLP for AADC binding. This could also explain the altered K_m apparent for PLP.

The vitamin B_6 antagonist 4-DP when added to the PLP deficient SH-SY5Y cells induced no further reduction in intracellular PLP concentrations. However there were further losses in AADC activity and protein. It has previously been demonstrated that administration of 4-DP to rats does not alter PLP availability (Bayoumi *et al*, 1972; Coburn *et al*, 1981). However 4-DP did reduce the availability of the PLP-dependent enzyme glutamic acid decarboxylase, whilst a vitamin B_6 deficient diet had no effect (Bayoumi *et al*, 1972). This indicates that the effects of PLP deficiency and those of 4-DP may act via different mechanisms. In the current study PLP-deficient 4-DP treated

cells were found to have reduced AADC mRNA expression at 72 hours which was not observed in PLP deficient cells that were not treated with 4-DP. This demonstrates that the difference between 4-DP treatment and PLP deficiency cells may relate to effects of 4-DP on AADC expression. Matsuda *et al* (2004) reported a loss of AADC activity and protein in response to 4-DP in combination with vitamin B_6 deficient medium in rat pheochromocytoma cells. The authors suggested that this loss was related to an increase in AADC degradation. However in the current study treatment of PLP deficient cells with 4-DP did not alter the stability of AADC over 8hr demonstrating that increased degradation may not be involved in this response. Furthermore the reduction in AADC expression observed here indicates that downregulation rather than increased degradation may, at least in part, be responsible for the effect of this treatment. In the current experiments 4-DP alone led to a loss of AADC activity with L-dopa as substrate similar to that seen with the 4-DP cells cultured in vitamin B_6 deficient media, however the effect of 4-DP alone on PLP availability, AADC protein or mRNA expression was not investigated. It could be important for future investigations to examine the effect of 4-DP treatment in cells that are not cultured in vitamin B_6 deficient media to provide an improved understanding of the effect of this chemical upon AADC and PLP availability.

4-DP can be phosphorylated by pyridoxal kinase to 4-deoxypyridoxine 5'-phosphate (Hurwitz, 1955). 4-deoxypyridoxine 5'-phosphate has been shown to compete with PLP binding in the active site of some PLP-dependent enzymes, thereby acting as a competitive inhibitor for PLP but a non-competitive inhibitor for the substrate (Meister *et al*, 1953; Hurwitz, 1955; Amer *et al*, 1967). A reduced V_{max} but unaltered K_m such as that observed here in 4-DP treated PLP deficient cells could indicate a kinetic change resulting from inhibitor that is non-competitive for the substrate. However a decrease in active enzyme would also result in a reduced V_{max} and unaltered K_m which would also be consistent with the observed reduction of AADC protein and expression. Consequently it seems unlikely that inhibition alone can account for the loss in AADC activity. It has previously been observed that PLP can interact with transcription factors leading to alterations in expression (Allgood *et al*, 1993; Huq *et al*, 2007). Potentially 4-DP or its phosphorylated derivative could act as an inhibitor for the interaction of PLP with a transcription factor and that the inhibition of this interaction led to the observed decrease in AADC expression. The ability of a PLP antagonist to reduce AADC

expression may potentially indicate that PLP is involved in the control of AADC expression. This effect may not have been apparent in the PLP deficient cells as intracellular PLP concentrations were only reduced by one-third. Conversely it is also possible that the observed alteration in AADC expression relates to a nonspecific action of 4-DP rather than an effect of PLP antagonism.

The proportion of AADC activity lost with either L-dopa or 5-HTP as substrate was almost equal in the current study in response to PLP deficiency or 4-DP treatment. However in vitamin B_6 deficient rodents it has been shown that in some tissues the loss of L-dopa decarboxylase and 5-HTP decarboxylase activities were not equivalent (Rahman *et al*, 1982; Siow and Dakshinamurti, 1985). One potential explanation for this previously observed difference could relate to post-translational modification of AADC. AADC has been demonstrated to be phosphorylated by both cAMP and cyclic guanosine monophosphate (cGMP) -dependent protein kinases in a manner that increases AADC L-dopa decarboxylase activity (Duchemin *et al*, 2000; Duchemin *et al*, 2010). It is possible that the phosphorylation of AADC could induce alterations in its structure allowing one substrate to be favoured over another. This could explain possible differences in AADC substrate preference between individual tissues. However despite this hypothetical difference the expected overall effect of a loss of AADC, either due to PLP deficiency or AADC deficiency, would be to decrease both dopamine and serotonin metabolism by a relatively equivalent level.

6.7.2 Clinical consequences of the effect of PLP on AADC

Patients with AADC deficiency are thought to retain a very low level of AADC activity. In the current study five out of six patients had a detectable level of plasma AADC activity. Similarly in previous studies the majority of AADC deficient patients have been shown to have a detectable level of AADC activity (Verbeek *et al*, 2007; Manegold *et al*, 2009). This small amount of AADC activity may be maintaining a basal level of monoamines within these patients, and AADC would almost certainly be rate limiting for monoamine production in this situation. In support of this in the current study some reductions in catecholamine production were seen, following addition of L-dopa, in PLP deficient cells and as well as those cells treated with 4-DP. The PLP deficient cells in the present study still maintained two-thirds the level of PLP compared to control however there was still a loss of AADC activity and protein.

Furthermore a nearly equivalent proportion of AADC activity and PLP were lost, indicating that AADC may be sensitive to PLP deficiency. It is possible therefore that if patients with AADC deficiency became even mildly deficient in PLP then the already low level of AADC could be further reduced. This situation could possibly lead to a worsening of clinical symptoms due to a reduction in residual monoamine production. Most patients with AADC deficiency are treated with either pyridoxine or PLP, although for the majority of patients this treatment does not lead to improvements in clinical condition (Pons *et al*, 2004; Lee *et al*, 2008a; Brun *et al*, 2010). However it is possible that vitamin B_6 supplementation rather than acting to increase AADC activity may operate as more of a preventative treatment against reductions in AADC that would result from low levels of PLP. It should also be noted that in a recent survey of patients with AADC deficiency 29% of patients were not treated with either pyridoxine or PLP (Brun *et al*, 2010). In these cases it may be important to monitor PLP status to ensure that these patients do not become PLP deficient.

L-dopa is used in the treatment of several inherited metabolic conditions including tyrosine hydroxylase deficiency and disorders of tetrahydrobiopterin metabolism (Hyland, 2007; Pearl *et al*, 2007). Furthermore L-dopa is also administered to patients with Parkinson's disease (Schapira *et al*, 2009). This treatment is reliant on AADC within the CNS to convert L-dopa into dopamine. Consequently the efficacy of L-dopa treatment may also be dependent on the availability of central PLP, not only as a coenzyme for AADC but also to maintain the availability of the AADC enzyme. If the availability of PLP was reduced in L-dopa treated patients then there could potentially be a resultant decrease in AADC and consequently this would reduce the conversion of administered L-dopa into dopamine. Certainly PLP-deficient cells treated with 4-DP demonstrated reduced L-dopa metabolism, and PLP-deficient cells without 4-DP also showed reduced extracellular dopamine, compared to control, following addition of L-dopa. Furthermore this potential effect could be particularly important in patients treated with L-dopa due to a Pictet-Spengler reaction of L-dopa and PLP producing a tetrahydroisoquinoline derivative (see figure 6.10; Evered, 1971). Therefore L-dopa treatment may directly reduce PLP availability. This reaction may also be important in AADC deficiency where L-dopa accumulates due to the metabolic block at AADC. Consequently determining PLP status could be important for the monitoring of patients with AADC deficiency and also in other conditions when attempting to optimise L-dopa

treatment. This could be analogous to the need to monitor 5-methyltetrahydrofolate (5-MTHF) status in AADC deficiency and L-dopa treatment, where methylation of the high levels of L-dopa by COMT can deplete 5-MTHF (Surtees and Hyland, 1990; Brautigam *et al*, 2000).

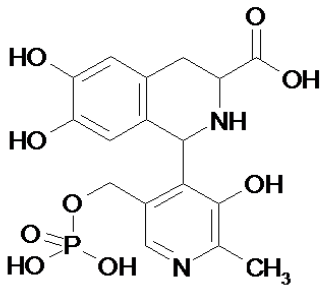


Figure 6.10 L-dopa-pyridoxal 5'-phosphate Pictet Spengler adduct.

In Parkinson's disease patients treated with L-dopa the administration of pyridoxine has been demonstrated to reverse the clinical effect of L-dopa (Duvoisin *et al*, 1969). However when L-dopa is co-administered with a peripheral AADC inhibitor such as benserazide or carbidopa, pyridoxine does not reverse the clinical effect of this treatment (Klawans *et al*, 1971). This suggests that pyridoxine administration increases peripheral AADC activity, leading to increased breakdown of L-dopa peripherally. In the current study increasing PLP availability to SH-SY5Y cells did not lead to significant changes in AADC. However this treatment also failed to increase intracellular concentrations of PLP and consequently the effect of increased PLP availability on AADC requires further investigation. Nonetheless if pyridoxine therapy was considered as a treatment for any patient receiving L-dopa therapy then it would be essential that this patient were also treated with a peripheral AADC inhibitor to prevent pyridoxine reversing the clinical effect of L-dopa. However pyridoxine treatment has been suggested to be beneficial for some Parkinson's disease patients who have a particular genetic variant of the COMT gene (Tan *et al*, 2005). Additionally higher dietary intake of vitamin B_6 has been suggested to decrease the risk of Parkinson's disease, although only for smokers (de Lau *et al*, 2006). Furthermore a recent study has linked a single nucleotide polymorphism in the pyridoxal kinase gene with Parkinson's

disease, indicating that PLP availability could in some cases play a role in the aetiology of Parkinson's disease (Elstner *et al*, 2009). Whether alterations in AADC availability are associated with these observations is unknown, however as this would directly relate vitamin B_6 to dopaminergic neurons that degenerate in Parkinson's disease, this potential connection may be worthy of further investigation.

Patients with PNPO deficiency present with neonatal seizures that are responsive to PLP treatment but not to pyridoxine (Mills *et al*, 2005; Hoffmann *et al*, 2007). Monoamine metabolites in the CSF of patients with PNPO deficiency have been reported to be variable. With decreases in both HVA and 5-HIAA coupled with increases in 3-OMD reported in some cases, similar to that seen in AADC deficiency (Brautigam *et al*, 2002; Mills *et al*, 2005; Hoffmann *et al*, 2007). In the present study decreases in AADC activity were found in the plasma of two patients with PNPO deficiency. Consequently both of the biochemical hallmarks of AADC deficiency, CSF findings and plasma AADC activity, can be similarly altered in PNPO deficiency. Therefore there is the potential for confusion, at least at the biochemical level, when diagnosing these conditions. As seizures have also been reported in AADC deficiency there is also the possibility of some overlap in clinical phenotype between these two conditions (Swoboda *et al*, 2003; Hsieh *et al*, 2005; Anselm and Darras, 2006; Ito *et al*, 2008). In consequence to ensure a full distinction can be made between these two conditions it could be important to determine PLP status, which should be very low in PNPO deficiency as well as glycine and threonine levels in CSF and urine which may be elevated in PNPO deficiency but not AADC deficiency (Mills *et al*, 2005; Hoffman *et al*, 2007). Conversely HVA has been demonstrated to be normal or variable in some cases of PNPO deficiency (Hoffman *et al*, 2007). It is interesting to note therefore that in PLP-deficient cells treated with 4-DP that HVA was unaffected despite significant reductions in both dopamine and DOPAC. This potentially indicates, albeit in a greatly simplified system, that the loss of dopamine may need to be quite severe before alterations are seen in the levels of HVA.

Drug-induced Parkinsonism and Tardive dyskinesia are common and often persistent side-effects of neuroleptic medication (reviewed by Haddad and Dursun, 2008). Several individual case reports and one small scale clinical trial have found improvements in these side effects following administration of pyridoxine (DeVeaugh-

Geiss and Manion 1978; Sandyk and Pardeshi, 1990; Lerner *et al*, 1999; Lerner *et al*, 2007). In one case report the authors proposed that the effect of pyridoxine treatment was related to increases in AADC activity, which in turn enhanced dopamine and serotonin production (Sandyk and Pardeshi, 1990). In the current study treatment of cells with PLP failed to alter intracellular PLP concentrations or AADC activity, and so it remains unknown if increasing intracellular PLP availability can increase AADC activity. However deficiency of PLP could potentially be a risk factor for drug-induced movement disorders and consequently those patients that are responsive to pyridoxine treatment may have an underlying PLP deficiency (Sandyk and Pardeshi, 1990; Miodownik *et al*, 2008). It is possible that this PLP deficiency would lead to a reduction in AADC and therefore in this circumstance increasing intracellular PLP availability may increase AADC levels, and this could be involved in the improvement of side-effects in these cases.

6.8 Conclusions

The data presented in this chapter would suggest that PLP deficiency such as occurs in PNPO deficiency can lead to a loss of active AADC. This loss is unlikely to be related to a decrease in enzyme stability or to increased degradation. As 4-DP decreased AADC mRNA levels it is possible that AADC expression can be influenced by PLP. However PLP deficiency in SH-SY5Y cells did not alter AADC expression, but still reduced AADC activity and protein, indicating an alternative mechanism for the loss of active AADC. The effect of PLP deficiency on AADC demonstrates the potential importance of maintaining adequate PLP availability in patients with AADC deficiency, to ensure that residual AADC is not further depleted by PLP deficiency. Likewise this observation may have implications for L-dopa therapy, as a loss of AADC due to decreased PLP availability may decrease L-dopa efficacy. Additionally determining the PLP status of patients when plasma AADC activity is measured may be useful for the biochemical distinction of AADC deficiency and PNPO deficiency. Finally the potential benefit of pyridoxine treatment for patients with neuroleptic-induced movement disorders may relate to the relief of PLP deficiency consequently leading to an increase in AADC levels.

Chapter 7

General Discussion

AADC deficiency results in a deficit of dopamine and serotonin, as well as an accumulation of their precursors L-dopa and 5-HTP respectively. The resultant clinical phenotype affects central neurological systems and peripheral functions and in the majority of cases response to treatment is poor (Swoboda *et al*, 1999; Swoboda *et al*, 2003; Pons *et al*, 2004; Brun *et al*, 2010). The first aim of this study was to establish the plasma AADC activity assay as a diagnostic test within the UK. This assay which is described in chapter 3 (see section 3.5.1) is now established within the Neurometabolic Unit (National Hospital, Queen Square, London) and has been used in the diagnosis of six new cases of AADC deficiency.

Imaging results from the brains of patients with AADC deficiency are suggestive of neurodegeneration in some cases (Lee *et al*, 2009; Manegold *et al*, 2009; Brun *et al*, 2010). Consequently during this study the hypotheses that L-dopa and 5-HTP accumulation lead to alterations in antioxidant status, damage to the mitochondrial respiratory chain and neuronal cell death were investigated in neuronal-like and astrocyte-like cell culture. L-dopa was found to be toxic to neuronal-like cells, however this toxicity was only observed at >250µM. Additionally the L-dopa metabolite 3-OMD that accumulates to a greater extent than L-dopa was not found to be toxic to neuronal cells at concentrations <500µM. This implicates COMT, the enzyme responsible for the methylation of L-dopa, as an important step in the detoxification of accumulating L-dopa. 5-HTP alone was found to be both growth inhibitory and induce some cell lysis of neuronal-like cells, although this effect was only observed at 5-HTP concentrations >1000µM.

The level of L-dopa or 5-HTP required for the observed effects are above the level expected in the brain of patients with AADC deficiency, and are certainly above the level in patient CSF. However in this study only acute effects rather than chronic effects were observed. In AADC deficient patients neurons would be continually exposed to L-dopa and 5-HTP. It is possible that this persistent exposure would have a cumulative effect that would be detrimental to neurons over a longer time period. Both L-dopa and 5-HTP are used as pharmacological treatments in a range of neurological conditions (Ramaekers *et al*, 2001; Turner *et al*, 2006; Hyland, 2007; Pearl *et al*, 2007; Longo *et al*, 2009; Schapira *et al*, 2009). As treatments generally neither L-dopa nor 5-HTP are considered to be neurotoxic (Hefti *et al*, 1981; Nardini *et al*, 1983; Perry *et al*,

1984; Quinn *et al*, 1986; Meltzer *et al*, 1997; Zeng *et al*, 2001; Mytilineou *et al*, 2003; Turner *et al*, 2006). However there may be an important fundamental difference between drug administration and endogenous accumulation. Administered 5-HTP and L-dopa would be taken up by appropriate neuronal systems, converted into their respective neurotransmitters and packaged into vesicles. In AADC deficiency 5-HTP and L-dopa would be produced within neurons and due to the deficiency in conversion to dopamine and serotonin would then be free within the cytosol. Increased cytosolic dopamine has been found to lead to neurodegeneration in rats (Caudle *et al*, 2007; Chen *et al*, 2008a) and in the current study L-dopa was found to be similarly toxic to dopamine and consequently cytosolic accumulation of L-dopa may also lead to neurodegeneration. In support of this expression of AADC and VMAT2 were found to protect neurons against L-dopa toxicity (Doroudchi *et al*, 2005).

This thesis investigated the effect of L-dopa and 5-HTP on isolated neuronal-like cells. It is possible that other cell types may influence the toxicity of these metabolites. It was hypothesised that the kynurenine pathway, an alternative metabolic route for 5-HTP metabolism, led to the production of toxic metabolites in AADC deficiency. The effect of 5-HTP in isolated astrocyte-like cells was investigated and was found to have a small effect on cell lysis only at a concentration of 1000µM and in the presence of IFN-γ, an activator of the kynurenine pathway. This could indicate some toxicity of 5-HTP to astrocyte-like cells when the kynurenine pathway is activated, however the activity of the kynurenine pathway was not measured in these cells. IFN-γ also influenced the effects of 5-HTP on neuronal-like cells leading to cell loss although not cell lysis. This could suggest that IFN-γ and 5-HTP treatment induced some toxicity, although other explanations are also possible (see section 4.6.2). 5-HTP and serotonin concentrations were not decreased by IFN-γ treatment which may suggest that the kynurenine pathway may have not been activated by IFN-γ in neuronal-like cells. Other cell types within the brain particularly microglial cells have been implicated as being involved in kynurenine metabolism (Guillemin *et al*, 2005b). It seems plausible therefore that metabolic interactions between different cell types may alter the toxicity of 5-HTP.

It is possible that together L-dopa and 5-HTP could have a cumulative effect. For example the auto-oxidation of L-dopa within the cytosol of dopaminergic neurons could lead to neurodegeneration. Neurodegeneration can lead to immune activation of

microglial cells and astrocytes, which can include induction of IDO the rate-limiting enzyme in the kynurenine pathway (Heyes *et al*, 1993; Kreutzberg, 1996; Alberti-Giani and Cesura, 1998; Mrak and Griffin, 2005; Amor *et al*, 2010). Hypothetically accumulated 5-HTP could then be converted via the kynurenine pathway into a putative toxic metabolite which could then inflict a secondary insult. Similarly immune activation and production of the kynurenine metabolite quinolinic acid has been linked to the progression and severity of Huntington's disease (Whetsell and Schwarcz, 1989; Forrest *et al*, 2010; Sathyasaikumar *et al*, 2010; Schwarcz *et al*, 2010). In particular quinolinic acid has been demonstrated to induce excitotoxicity through activation of glutamatergic NMDA receptors leading to neurodegeneration (Stone and Perkins, 1981; Foster *et al*, 1983; Beal *et al*, 1986). Further work is needed first to assess whether 5-HTP-kynurenine metabolism can lead to the production of toxic metabolites. In the current study IFN-γ was found to influence the effects of 5-HTP on cell growth, however it was unclear whether IDO was active in neuronal-like cells. Secondly additional experiments are required to investigate whether there are interactions between L-dopa and 5-HTP which may be deleterious to patients with AADC deficiency.

During this study it was demonstrated that dopamine and to a lesser extent serotonin were able to increase the availability of GSH within neuronal-like cells. GSH plays an important role in the detoxification of free radicals and H_2O_2 (reviewed by Martin and Teismann, 2009; Ballatori *et al*, 2009), consequently both dopamine and serotonin may act to enhance antioxidant defence within neurons. Therefore due to dopamine and serotonin deficits the neurons of patients with AADC deficiency could potentially have a lowered antioxidant status and may be more susceptible to oxidative stress. In addition GSH status has previously been demonstrated to be an important factor during apoptosis (Froissard *et al*, 1997; Hou et al, 1997; Nicole *et al*, 1998). Depletion of cellular GSH has been shown to either initiate or potentiate apoptosis, whilst increased expression of γ-glutamylcysteine ligase can increase resistance to apoptosis (Froissard *et al*, 1997; Nicole *et al*, 1998; Armstrong *et al*, 2004; Botta *et al*, 2004; Diaz-Hernandez *et al*, 2005). AADC deficiency is also likely to result in a deficiency in melatonin a product of serotonin produced mainly in the pineal gland (reviewed by Maronde and Stehle, 2007). In addition to being a neurohormone melatonin has been demonstrated to act as an antioxidant with greater potency than serotonin and other related indolic compounds

(Poeggeler *et al*, 2002; Hardeland *et al*, 1993; Tan *et al*, 1994). Furthermore increased lipid peroxidation, GSH depletion and decreased superoxide dismutase activity observed in the brain of a mouse model of Alzheimer's disease expressing a mutant amyloid precursor protein (APP) could be prevented by administration of melatonin (Feng *et al*, 2006). Therefore it is possible that the absence of melatonin in AADC deficient patients could also reduce brain antioxidant status. Further work will be required particularly direct clinical or pathological observations may be important in determining the role of decreases antioxidant status in AADC deficiency. If this were to be involved then it is possible that pharmacological treatment with antioxidants or neuroprotective agents could be of benefit in AADC deficiency. For example the compound AEOL 10150, a metallo-porphyrin which catalyses the reduction of $O_2^{\bullet-}$, H_2O_2 and $ONOO^{\bullet-}$, is undergoing clinical trials for treatment of the neurodegenerative disorder amyotrophic lateral sclerosis (Traynor *et al*, 2006; Scatena *et al*, 2007; Chen *et al*, 2008b). Additionally the MAO inhibitor selegiline is thought to elicit some neuroprotection that is unrelated to inhibition of MAO (Maruyama *et al*, 2002; Magyar *et al*, 2006; Naoi *et al*, 2007).

Dopamine as well as increasing GSH within neuronal-like cells was also demonstrated in the current study to increase GSH release from astrocyte-like cells. Astrocytic release of GSH is considered to be the first stage of GSH trafficking from astrocytes to neurons (see section 1.7.6 and figure 1.9; Dringen *et al*, 1999; Stewart *et al*, 2002). Consequently dopamine may play a dual role in increasing neuronal GSH status, firstly by increasing GSH synthesis within neurons and secondly by increasing the availability of substrates for GSH synthesis through increased trafficking from astrocytes. It is possible that dopamine treatment led to activation of the Nrf2/keap1 signalling pathway, which in turn led to upregulation of genes that can be controlled by the ARE promoter sequence (see section 5.6.3; Rushmore *et al*, 1991; Venugopal *et al*, 1996; Itoh *et al*, 1997; Itoh *et al*, 1999; Zipper and Mulcahy, 2002; Itoh *et al*, 2003). It was notable that whilst dopamine increased GSH, L-dopa in the absence of AADC activity was unable to elicit such an effect. This suggests that dopamine provides a specific signal for upregulation of GSH synthesis and trafficking. Both serotonin and 5-HTP treatment also induced increases in GSH levels within neuronal-like cells, although this effect was much smaller than that observed with dopamine. It is possible that the increase in GSH

following 5-HTP or serotonin treatment was related to the ability of indolic compounds to act as reducing agents (Herraiz and Galisteo, 2004; Munoz-Castaneda *et al*, 2006).

GSH plays a particularly important role in maintaining mitochondrial redox balance and preventing oxidative damage to the respiratory chain (Bolanos *et al*, 1994; Heales *et al*, 1995; Bolanos *et al*, 1996). Mitochondrial respiratory chain complexes I and III produce $O_2^{\bullet-}$ which must be detoxified to avoid oxidative damage to proteins within the mitochondrial matrix (Kudin *et al*, 2005). Furthermore the respiratory chain complexes are known to be targets for oxidative damage (Zhang *et al*, 1990; Benzi *et al*, 1991). In the current study L-dopa or 5-HTP treatment of neuronal-like cells did not lead to any loss of activity of the mitochondrial respiratory chain complexes. This indicates that oxidative damage to the respiratory chain did not occur in response to these treatments. It is possible that a chronic exposure to L-dopa or 5-HTP could eventually lead to damage to the respiratory chain although this will require further investigation. Dopamine treatment led to an increase in complex I activity, whilst 5-HTP and serotonin treatment may have led to an increase in complex II/III activity. These increase could relate to increased levels of GSH decreasing basal ROS in turn allowing electron transport to operate at a more efficient level or alternatively to an upregulation of mitochondrial complex expression (see section 5.6.4).

Vitamin B_6, usually in the form of pyridoxine but also sometimes as PLP, has been used as a treatment in AADC deficiency since the first two described cases (Hyland and Clayton, 1990; Brun *et al*, 2010). This treatment has not been reported to improve clinical symptoms in the majority of cases (Hyland *et al*, 1992; Korenke *et al*, 1997; Maller *et al*, 1997; Swoboda *et al*, 1999; Pons *et al*, 2004). In the current study the involvement of PLP availability in maintaining active AADC was investigated. It was demonstrated that in PLP deficiency AADC activity was reduced in patients and in cell culture. In cell culture this deficit was related to a loss of protein, although not to changes in stability or expression (see section 6.7.1). This work demonstrates the level of active AADC is dependent on appropriate PLP availability. Similar results have been demonstrated previously in rodents and PC12 cells, although the mechanism for this effect has not previously been investigated (Rahman *et al*, 1982; Siow and Dakshinamurti, 1985; Guilarte *et al*, 1987; Matsuda *et al*, 2004). The majority of patients with AADC deficiency are thought to maintain a residual level of AADC

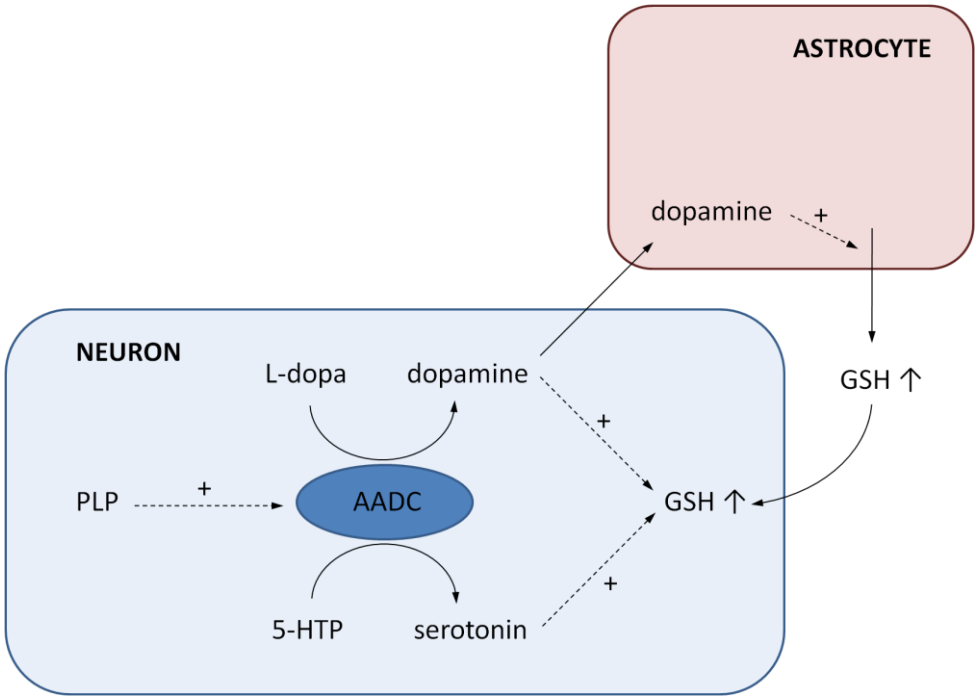


Figure 7.1 Summary of results. In AADC deficiency it is possible that GSH status in neurons may be impaired due to the potential positive effects of dopamine and serotonin on GSH status. Pyridoxal 5'-phosphate (PLP) may be important in maintaining the level of active AADC consequently in AADC deficiency maintaining PLP levels could be an important therapeutic strategy to avoid loss of residual AADC activity. AADC: aromatic L-amino acid decarboxylase, GSH: reduced glutathione, 5-HTP: L-5-hydroxytryptophan, L-dopa: L-3,4-dihydroxyphenylalanine.

activity, which could be preserving a basal level of monoamines. Both a low level of plasma AADC activity and CSF HVA and 5-HIAA have been detected in the majority of patients (see figure 6.3; Verbeek *et al*, 2007; Manegold *et al*, 2009; Brun *et al*, 2010). Reductions in PLP levels led to a concomitant decrease in active AADC and therefore in AADC deficiency prevention of PLP deficiency may be an important therapeutic aim. Patients with AADC deficiency could be at increased risk of PLP deficiency due to a potential reaction between PLP and accumulated L-dopa forming a PLP-L-dopa Pictet-Spengler adduct (Evered, 1971). Additionally feeding difficulties have been reported in some cases of AADC deficiency (Pons *et al*, 2004), which could potentially lead to a dietary deficiency of PLP. Consequently the evidence presented in this thesis (see section 6.6) provides a potential justification for the continued use of vitamin B_6 treatment in AADC deficiency. Although this treatment may not improve clinical symptoms in AADC deficiency, potentially it could prevent PLP deficiency and any associated loss of residual active AADC.

Whilst decreasing PLP availability led to a loss of active AADC, increasing PLP availability in cell culture did not lead to an increase in AADC. However PLP was not increased intracellularly following PLP treatment and consequently the effect of increased PLP availability on AADC requires further investigation. A range of treatments have been demonstrated to increase AADC activity and availability in rodent brain (Boomsma *et al*, 1989; Zhu *et al*, 1992; Hadjiconstantinou *et al*, 1993; Cho *et al*, 1999; Duchemin *et al*, 2000; Neff *et al*, 2006; Duchemin *et al*, 2010). Direct phosphorylation of AADC by both cAMP and cGMP-dependent protein kinases has been reported (Duchemin *et al*, 2000; Duchemin *et al*, 2010). Furthermore AADC appears to be regulated by a feedback mechanism such that treatment with dopamine or serotonin receptor antagonists or AADC inhibitors leads to an upregulation of AADC (Boomsma *et al*, 1989; Zhu *et al*, 1992; Hadjiconstantinou *et al*, 1993; Cho *et al*, 1999; Neff *et al*, 2006). Increased plasma AADC activity has been reported in Parkinson's disease patients treated with L-dopa and a peripheral AADC inhibitor (Boomsma *et al*, 1989). A potential future treatment of AADC deficiency could aim to harness AADC regulation to maximise the residual level of AADC activity in patients and possibly improve monoamine production (see Allen *et al*, 2009). For example the antipsychotic clozapine, which antagonises D2-like dopamine receptors and serotonin receptors, can increase striatal AADC activity 40-50% in mouse (Neff *et al*, 2006). Dopamine and serotonin antagonists are likely to exacerbate the symptoms of AADC deficiency

through blockade of dopamine and serotonin receptors. However a drug screening study could reveal other compounds which can similarly increase AADC activity without inhibiting monoamine systems.

An alternative to pharmacological treatment could be to use gene therapy to re-introduce the human AADC gene into the brains of patients with AADC deficiency. Gene transfer is achieved by using recombinant viral vectors such as adeno-associated viruses (AAVs). A gene therapy approach using the human AADC gene delivered using an AAV has been developed for Parkinson's disease treatment (Carlsson *et al*, 2007). The aim of this therapy is to increase AADC expression to extend the treatment window of L-dopa therapy. This approach has been reported to be successful in a primate model of Parkinson's disease and in a safety trial of five Parkinson's disease patients (Bankiewicz *et al*, 2006; Eberling *et al*, 2008). A similar system could be used to re-introduce the AADC gene into selected brain regions of patients with AADC deficiency. Indeed a gene therapy trial using the human AADC gene is currently underway in Taiwan where one patient with AADC deficiency has been treated (http://www.wretch.cc/blog/aadc/33573305). It is unclear however how a brain that has developed in the near absence of monoamines will react to their production later in life. An improved understanding of the pathogenesis of AADC deficiency, which the current study has begun to investigate, will hopefully lead to new treatment targets and improved medication for this debilitating and often intractable disorder.

7.1 Conclusions

Evidence presented in this thesis indicates that L-dopa and 5-HTP are capable of inducing some neuronal toxicity in cell culture although only at concentrations above that seen in AADC deficiency. However it is possible that this toxicity could become more potent during prolonged exposure as would be the case in patients. The enzyme COMT may protect against L-dopa toxicity by converting L-dopa to the non-toxic metabolite 3-OMD. Dopamine deficiency could potentially lead to impairment of antioxidant signalling resulting in decreased GSH in neurons and decreased GSH release from astrocytes. Serotonin deficiency may also lead to a decrease in GSH levels within neurons. Vitamin B_6 treatment should be considered as a preventative treatment against PLP deficiency in AADC deficient patients and leading to a consequent loss of residual AADC enzyme activity. In AADC deficient patients not receiving vitamin B_6 supplementation monitoring of PLP status could be important. Future treatments of

AADC deficiency could aim to increase AADC activity either by pharmacological strategies to maximise residual activity or through gene therapy.

7.2 Further work

The results of this thesis were obtained from neuroblastoma and astrocytoma cells and AADC deficiency was modelled using the chemical AADC inhibitor NSD-1015. In order to confirm and extend these results other models of AADC deficiency and direct measurement of patient samples should be employed including:

- A knock down animal model.
- Direct clinical or pathological evidence of neurodegeneration and reduced antioxidant status.
- Patient CSF analysis to determine GSH status and identify degenerative markers and indicators of oxidative stress.

IFN-γ treatment was used in this thesis to activate IDO the rate limiting enzyme in the kynurenine pathway. Whilst IFN-γ treatment was found to alter the response of neuroblastoma cells to 5-HTP it was not directly determined whether the kynurenine pathway was active. This work could be extended by:

- Measuring IDO activity and quinolinic acid levels in neuroblastoma and astrocytoma cells.
- Examining the effect of microglial cells upon 5-HTP metabolism and toxicity using a co-culture system with both microglial and neuronal cells (Bolanos *et al*, 1996; Stewart *et al*, 1998; Stewart *et al*, 2000; Gegg *et al*, 2005).
- Determining the metabolic fate of accumulated 5-HTP by adding a stable isotope [^{13}C-3]-5-HTP to cell culture and identifying the resultant metabolites by mass spectrometry (Zamboni *et al*, 2009; Hiller *et al*, 2010).
- Investigating the levels of kynurenine metabolites in the CSF of AADC deficient patients.

Dopamine treatment was found to increase GSH levels within neuroblastoma cells and GSH release from astrocytoma cells. This could suggest activation of the Nrf2/keap1

signalling pathway. It could be of interest to examine whether this pathway was activated by dopamine treatment. This could be invstigated by:

- Determining whether Nrf2 locates to the nucleus using confocal microscopy or whether the Nrf2/keap1 complex is disrupted by a co-immunoprecipitation study (Zipper and Mulcahy, 2002; Itoh *et al*, 2003).

- Determining the activity of other enzymes, such as NQO1, that are regulated by Nrf2 following dopamine treatment.

Additionally a co-culture system (Bolanos *et al*, 1996; Stewart *et al*, 1998; Stewart *et al*, 2000; Gegg *et al*, 2005) could be employed to examine whether increased GSH release from astrocytomas results in increased GSH within neurons following dopamine treatment. To further establish the physiological relevance of these observations the effect of dopamine treatment on GSH in primary rat neurons and astrocytes could also be investigated.

In this thesis reduced levels of PLP were found to decrease the availability of active AADC in patients and in cell culture. The mechanism for this response was found not to relate to expression or stability. Alternative mechanisms could involve alterations in dimerisation and phosphorylation. This work could be extended by:

- Investigating AADC dimerisation using blue-native PAGE to determine the ratio of monomer to dimer (Wittig *et al*, 2006).

- Determining phosphorylation status of AADC following PLP deficiency using a phospho-antibody raised against phosphorylated AADC (Goto and Inagaki, 2007).

- Further investigating whether increasing intracellular PLP concentrations can increase AADC activity potentially by overexpression of the PNPO gene within cells.

- An siRNA knock down strategy (Cullen, 2005) against PNPO could also be employed to reduce intracellular PLP further to determine the effect of a greater level of PLP deficiency.

- A long-term study could be conducted to determine whether raised levels of L-dopa can affect PLP status.

- Monitoring PLP status in patients with AADC deficiency not treated with vitamin B_6 could improve understanding of whether PLP deficiency is a potential concern in this condition.

Further work may also need to be conducted examining the effects of 4-DP. In this thesis 4-DP was used in combination with vitamin B_6 deficient medium mainly to allow comparison to a previous study (Matsuda *et al*, 2004). However to further understand the effects of this inhibitor it may be important to treat cells with 4-DP alone. This would allow the direct effects of 4-DP upon PLP metabolism and AADC expression to be investigated.

A new technology that could potentially be employed for the investigation of AADC deficiency is that of induced pluripotent stem (iPS) cells (Park *et al*, 2008a). Here somatic cells such as skin fibroblasts are reprogrammed into stem cell-like cells by the transfection of four transcription factors (Takahashi *et al*, 2007; Dimos *et al*, 2008; Park *et al*, 2008b). iPS cells can then be differentiated potentially into any cell type. Using cells from patients this technique could be used to make patient specific neurons. These neurons may perhaps be best used to perform drug screenings to investigate the potential of different compounds in the treatment of AADC deficiency. For example the ability of a range of available pharmaceuticals could be investigated to determine whether a treatment could increase AADC activity in patient derived cells.

References

Abdenur J. E., Abeling N., Specola N., Jorge L., Schenone A. B., van Cruchten A. C. and Chamoles N. A. (2006) Aromatic L-amino acid decarboxylase deficiency: unusual neonatal presentation and additional findings in organic acid analysis. *Mol. Genet. Metab.* **87,** 48-53.

Abeling N. G. G. M., van Gennip A. H., Barth P. G., van Cruchten A., Westra M. and Wijburg F. A. (1998) Aromatic L-amino acid decarboxylase deficiency: A new case with a mild clinical presentation and unexpected laboratory findings. *J. Inherit. Metab. Dis.* **21,** 240-242.

Abeling N. G. G. M., Brautigam C., Hoffmann G. F., Barth P. G., Wevers R. A., Jaeken J., Fiumara A., Knust A. and van Gennip A. H. (2000) Pathobiochemical implications of hyperdopaminuria in patients with aromatic L-amino acid decarboxylase deficiency. *J. Inherit. Metab. Dis.* **23,** 325-328.

Alberati-Giani D., Ricciardi-Castagnoli P., Kohler C. and Cesura A. M. (1996) Regulation of the kynurenine metabolic pathway by interferon-gamma in murine cloned macrophages and microglial cells. *J. Neurochem.* **66,** 996-1004.

Alberati-Giani D., Malherbe P., Ricciardi-Castagnoli P., Kohler C., Denis-Donini S. and Cesura A. M. (1997) Differential regulation of indoleamine 2,3-dioxygenase expression by nitric oxide and inflammatory mediators in IFN-gamma-activated murine macrophages and microglial cells. *J. Immunol.* **159,** 419-426.

Alberati-Giani D. and Cesura A. M. (1998) Expression of the kynurenine enzymes in macrophages and microglial cells: regulation by immune modulators. *Amino Acids* **14,** 251-255.

Albert V. R., Lee M. R., Bolden A. H., Wurzburger R. J. and Aguanno A. (1992) Distinct promoters direct neuronal and nonneuronal expression of rat aromatic L-amino acid decarboxylase. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12053-12057.

Allen G. F., Land J. M. and Heales S. J. (2009) A new perspective on the treatment of aromatic L-amino acid decarboxylase deficiency. *Mol. Genet. Metab.* **97,** 6-14.

Allen G. F., Neergheen V., Oppenheim M., Fitzgerald J. C., Footitt E., Hyland K., Clayton P. T., Land J. M. and Heales S. J. (2010) Pyridoxal 5'-phosphate deficiency causes a loss of aromatic L-amino acid decarboxylase in patients and human neuroblastoma cells, implications for aromatic L-amino acid decarboxylase and vitamin B6 deficiency states. *J. Neurochem.* **114,** 87-96.

Allgood V. E., Powell-Oliver F. E. and Cidlowski J. A. (1990) Vitamin B6 influences glucocorticoid receptor-dependent gene expression. *J. Biol. Chem.* **265,** 12424-12433.

Allgood V. E., Oakley R. H. and Cidlowski J. A. (1993) Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. *J. Biol. Chem.* **268,** 20870-20876.

Almeida A. and Medina J. M. (1998) A rapid method for the isolation of metabolically active mitochondria from rat neurons and astrocytes in primary culture. *Brain Res. Brain Res. Protoc.* **2,** 209-214.

Almeida A., Almeida J., Bolanos J. P. and Moncada S. (2001) Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 15294-15299.

Alvarez J. C., Bothua D., Collignon I., Advenier C. and Spreux-Varoquaux O. (1999) Simultaneous measurement of dopamine, serotonin, their metabolites and tryptophan in mouse brain homogenates by high-performance liquid chromatography with dual coulometric detection. *Biomed. Chromatogr.* **13,** 293-298.

Amer M. S., Abdel-Daim M. H. and Abdel-Tawab G. A. (1967) Studies with tryptophan metabolites in vitro. Kynurenine metabolism in liver homogenates of normal and Schistosoma mansoni-infested mice. *Biochem. J.* **104,** 656-662.

Amin A. H., Crawford T. B. B. and Gaddum J. H. (1954) The distribution of substance-P and 5-hydroxytryptamine in the central nervous system of the dog. *J. Physiol. (Lond).* **126,** 596-618.

Amor S., Puentes F., Baker D. and van d., V (2010) Inflammation in neurodegenerative diseases. *Immunology* **129,** 154-169.

Anselm I. A. and Darras B. T. (2006) Catecholamine toxicity in aromatic L-amino acid decarboxylase deficiency. *Pediatr. Neurol.* **35,** 142-144.

Armstrong J. S., Whiteman M., Yang H., Jones D. P. and Sternberg P., Jr. (2004) Cysteine starvation activates the redox-dependent mitochondrial permeability transition in retinal pigment epithelial cells. *Invest Ophthalmol. Vis. Sci.* **45,** 4183-4189.

Axelrod J. and Tomchick R. (1958) Enzymatic O-methylation of epinephrine and other catechols. *J. Biol. Chem.* **233,** 702-705.

Axelrod J. and Weissbach H. (1960) Enzymatic O-methylation of N-acetylserotonin to melatonin. *Science* **131,** 1312.

Axelrod J. (1962) Purification and properties of phenylethanolamine-N-methyl transferase. *J. Biol. Chem.* **237,** 1657-1660.

Azmitia E. C. and Segal M. (1978) An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. *J. Comp Neurol.* **179,** 641-667.

Azmitia E. C. (2001) Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. *Brain Res. Bull.* **56,** 413-424.

Bach A. W., Lan N. C., Johnson D. L., Abell C. W., Bembenek M. E., Kwan S. W., Seeburg P. H. and Shih J. C. (1988) cDNA cloning of human liver monoamine oxidase A and B: molecular basis of differences in enzymatic properties. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4934-4938.

Badawy A. A. (1977) The functions and regulation of tryptophan pyrrolase. *Life Sci.* **21,** 755-768.

Ballatori N., Krance S. M., Notenboom S., Shi S., Tieu K. and Hammond C. L. (2009) Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* **390,** 191-214.

Bankiewicz K. S., Forsayeth J., Eberling J. L., Sanchez-Pernaute R., Pivirotto P., Bringas J., Herscovitch P., Carson R. E., Eckelman W., Reutter B. and Cunningham J. (2006) Long-term clinical improvement in MPTP-lesioned primates after gene therapy with AAV-hAADC. *Mol. Ther.* **14,** 564-570.

Bartholini G., Burkard W. P. and Pletscher A. (1967) Increase of cerebral catecholamines caused by 3,4-dihydroxyphenylalanine after inhibition of peripheral decarboxylase. *Nature* **215,** 852-853.

Basbaum A. I. and Fields H. L. (1979) The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. *J. Comp Neurol.* **187,** 513-531.

Basma A. N., Morris E. J., Nicklas W. J. and Geller H. M. (1995) L-dopa cytotoxicity to PC12 cells in culture is via its autoxidation. *J. Neurochem.* **64,** 825-832.

Baumgarten H. G. and Grozdanovic Z. (1997) Anatomy of central serotoninergic projection systems, in *Serotoninergic neurons and 5-HT receptors in the CNS,* (Baumgarten H. G. and Gothert M., eds),Vol. 129, pp. 41-90. Springer-Verlag, Berlin.

Bayoumi R. A., Kirwan J. R. and Smith W. R. (1972) Some effects of dietary vitamin B6 deficiency and 4-deoxypyridoxine on γ-aminobutyric acid metabolism in rat brain. *J. Neurochem.* **19,** 569-576.

Beadle G. W., Mitchell H. K. and Nyc J. F. (1947) Kynurenine as an intermediate in the formation of nicotinic acid from tryptophane by Neurospora. *Proc. Natl. Acad. Sci. U. S. A.* **33,** 155-158.

Beal M. F., Kowall N. W., Ellison D. W., Mazurek M. F., Swartz K. J. and Martin J. B. (1986) Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* **321,** 168-171.

Beatty W. L., Belanger T. A., Desai A. A., Morrison R. P. and Byrne G. I. (1994) Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.* **62,** 3705-3711.

Ben Shachar D., Zuk R. and Glinka Y. (1995) Dopamine neurotoxicity: inhibition of mitochondrial respiration. *J. Neurochem.* **64,** 718-723.

Bengel D., Murphy D. L., Andrews A. M., Wichems C. H., Feltner D., Heils A., Mossner R., Westphal H. and Lesch K. P. (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol. Pharmacol.* **53,** 649-655.

Beninger R. J. and Hahn B. L. (1983) Pimozide blocks establishment but not expression of amphetamine produced environment specific conditioning. *Science* **220,** 1304-1306.

Beninger R. J., Cheng M., Hahn B. L., Hoffman D. C., Mazurski E. J., Morency M. A., Ramm P. and Stewart R. J. (1987) Effects of extinction, pimozide, sch 23390, and metoclopramide on food-rewarded operant responding of rats. *Psychopharmacology (Berl).* **92,** 343-349.

Benzi G., Curti D., Pastoris O., Marzatico F., Villa R. F. and Dagani F. (1991) Sequential damage in mitochondrial complexes by peroxidative stress. *Neurochem. Res.* **16,** 1295-1302.

Berman S. B. and Hastings T. G. (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. *J. Neurochem.* **73,** 1127-1137.

Bernardi P. and Petronilli V. (1996) The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J. Bioenerg. Biomembr.* **28,** 131-138.

Berry M. D., Juorio A. V., Li X. M. and Boulton A. A. (1996) Aromatic L-amino acid decarboxylase: A neglected and misunderstood enzyme. *Neurochem. Res.* **21,** 1075-1087.

Bertoldi M., Moore P. S., Maras B., Dominici P. and Voltattorni C. B. (1996) Mechanism-based inactivation of dopa decarboxylase by serotonin. *J. Biol. Chem.* **271,** 23954-23959.

Bertoldi M., Frigeri P., Paci M. and Voltattorni C. B. (1999) Reaction specificity of native and nicked 3,4-dihydroxyphenylalanine decarboxylase. *J. Biol. Chem.* **274,** 5514-5521.

Bertoldi M. and Voltattorni C. B. (2000) Reaction of dopa decarboxylase with L-aromatic amino acids under aerobic and anaerobic conditions. *Biochem. J.* **352,** 533-538.

Bertoldi M., Castellani S. and Voltattorni C. B. (2001) Mutation of residues in the coenzyme binding pocket of dopa decarboxylase. Effects on catalytic properties. *Eur. J. Biochem.* **268,** 2975-2981.

Bertoldi M. and Voltattorni C. B. (2009) Multiple roles of the active site lysine of dopa decarboxylase. *Arch. Biochem. Biophys.* **488,** 130-139.

Bickel H., Gerrard J. and Hickmans E. M. (1953) Influence of phenylalanine intake on phenylketonuria. *Lancet* **262,** 812-813.

Biedler J. L., Helson L. and Spengler B. A. (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* **33,** 2643-2652.

Biedler J. L., Rofflertarlov S., Schachner M. and Freedman L. S. (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res.* **38,** 3751-3757.

Bjorklund A. and Skagerberg G. (1982) Descending monoaminergic projections to the spinal cord, in *Brain stem control of spinal mechanisms,* (Sjolund B. and Bjorklund A., eds), pp. 55-88. Elsevier, Amsterdam.

Blainey J. D. and Gulliford R. (1956) Phenylalanine-restricted diets in the treatment of phenylketonuria. *Arch. Dis. Child.* **31,** 452-466.

Blakely R. D., Berson H. E., Fremeau R. T., Caron M. G., Peek M. M., Prince H. K. and Bradley C. C. (1991) Cloning and expression of a functional serotonin transporter from rat brain. *Nature* **354,** 66-70.

Blaschko H. (1942) The activity of l(-)-dopa decarboxylase. *J. Physiol. (Lond).* **101,** 337-349.

Blau N. (2004) Disorders of tetrahydrobiopterin metabolism, in *Diseases of Neurotransmission from bench to bedside,* (Hoffmann G. F., ed), pp. 143-152. SHS International, Heilbronn.

Bleijenberg B. G., Van Eijk H. G. and Leijnse B. (1971) The determination of non-heme iron and transferrin in cerebrospinal fluid. *Clin. Chim. Acta* **31,** 277-281.

Blessing H., Bareiss M., Zettlmeisl H., Schwarz J. and Storch A. (2003) Catechol-O-methyltransferase inhibition protects against 3,4-dihydroxyphenylalanine (DOPA) toxicity in primary mesencephalic cultures: new insights into levodopa toxicity. *Neurochem. Int.* **42,** 139-151.

Blundell J. E. and Latham C. J. (1980) Characterization of adjustments to the structure of feeding behavior following pharmacological treatment: effects of amphetamine and fenfluramine and the antagonism produced by pimozide and methergoline. *Pharmacol. Biochem. Behav.* **12,** 717-722.

Bolanos J. P., Peuchen S., Heales S. J., Land J. M. and Clark J. B. (1994) Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J. Neurochem.* **63,** 910-916.

Bolanos J. P., Heales S. J., Land J. M. and Clark J. B. (1995) Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. *J. Neurochem.* **64,** 1965-1972.

Bolanos J. P., Heales S. J., Peuchen S., Barker J. E., Land J. M. and Clark J. B. (1996) Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. *Free Radic. Biol. Med.* **21,** 995-1001.

Bonafe L., Thony B., Penzien J. M., Czarnecki B. and Blau N. (2001a) Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine neurotransmitter deficiency without hyperphenylalaninemia. *Am. J. Hum. Genet.* **69,** 269-277.

Bonafe L., Blau N., Burlina A. P., Romstad A., Guttler F. and Burlina A. B. (2001b) Treatable neurotransmitter deficiency in mild phenylketonuria. *Neurology* **57,** 908-911.

Bonner D. (1948) The identification of a natural precursor of nicotinic acid. *Proc. Natl. Acad. Sci. U. S. A.* **34,** 5-9.

Boomsma F., Vanderhoorn F. A. J. and Schalekamp M. A. D. H. (1986) Determination of aromatic L-amino acid decarboxylase in human plasma. *Clin. Chim. Acta* **159,** 173-183.

Boomsma F., Meerwaldt J. D., Tveld A. J. M. I., Hovestadt A. and Schalekamp M. A. D. H. (1989) Induction of aromatic L-amino acid decarboxylase by decarboxylase inhibitors in idiopathic parkinsonism. *Ann. Neurol.* **25,** 624-628.

Borowsky B., Adham N., Jones K. A., Raddatz R., Artymyshyn R., Ogozalek K. L., Durkin M. M., Lakhlani P. P., Bonini J. A., Pathirana S., Boyle N., Pu X. S., Kouranova E., Lichtblau H., Ochoa F. Y., Branchek T. A. and Gerald C. (2001) Trace amines: Identification of a family of mammalian G protein-coupled receptors. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8966-8971.

Bradbury M. W. (1997) Transport of iron in the blood-brain-cerebrospinal fluid system. *J. Neurochem.* **69,** 443-454.

Brautigam C., Wevers R. A., Hyland K., Sharma R. K., Knust A. and Hoffmann G. F. (2000) The influence of L-dopa on methylation capacity in aromatic L-amino acid decarboxylase deficiency: Biochemical findings in two patients. *J. Inherit. Metab. Dis.* **23,** 321-324.

Brautigam C., Hyland K., Wevers R., Sharma R., Wagner L., Stock G. J., Heitmann F. and Hoffmann G. F. (2002) Clinical and laboratory findings in twins with neonatal epileptic encephalopathy mimicking aromatic L-amino acid decarboxylase deficiency. *Neuropediatrics* **33,** 113-117.

Breisch S. T., Zemlan F. P. and Hoebel B. G. (1976) Hyperphagia and obesity following serotonin depletion by intraventricular para-chlorophenylalanine. *Science* **192,** 382-385.

Brenner-Lavie H., Klein E., Zuk R., Gazawi H., Ljubuncic P. and Ben Shachar D. (2008) Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: relevance to dopamine pathology in schizophrenia. *Biochim. Biophys. Acta* **1777,** 173-185.

Brenner-Lavie H., Klein E. and Ben Shachar D. (2009) Mitochondrial complex I as a novel target for intraneuronal DA: modulation of respiration in intact cells. *Biochem. Pharmacol.* **78,** 85-95.

Brown G. C., Bolanos J. P., Heales S. J. and Clark J. B. (1995) Nitric oxide produced by activated astrocytes rapidly and reversibly inhibits cellular respiration. *Neurosci. Lett.* **193,** 201-204.

Brun L., Ngu L. H., Keng W. T., Ch'ng G. S., Choy Y. S., Hwu W. L., Lee W. T., Willemsen M. A., Verbeek M. M., Wassenberg T., Regal L., Orcesi S., Tonduti D., Accorsi P., Testard H., Abdenur J. E., Tay S., Allen G. F., Heales S., Kern I., Kato M., Burlina A., Manegold C., Hoffmann G. F. and Blau N. (2010) Clinical and biochemical features of aromatic L-amino acid decarboxylase deficiency. *Neurology* **75,** 64-71.

Brunner H. G., Nelen M., Breakefield X. O., Ropers H. H. and Vanoost B. A. (1993a) Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase-A. *Science* **262,** 578-580.

Brunner H. G., Nelen M. R., Vanzandvoort P., Abeling N. G. G. M., Vangennip A. H., Wolters E. C., Kuiper M. A., Ropers H. H. and Vanoost B. A. (1993b) X-linked borderline mental-retardation with prominent behavioral disturbance: phenotype, genetic localization and evidence for disturbed monoamine metabolism. *Am. J. Hum. Genet.* **52,** 1032-1039.

Bruns D., Riedel D., Klingauf J. and Jahn R. (2000) Quantal release of serotonin. *Neuron* **28,** 205-220.

Bruyn R. P. and Stoof J. C. (1990) The quinolinic acid hypothesis in Huntington's chorea. *J. Neurol. Sci.* **95,** 29-38.

Bryson S. E., Drew G. M., Hall A. S., Ball S. G. and Balmforth A. J. (1992) Characterization of the dopamine receptor expressed by rat glomerular mesangial cells in culture. *Eur. J. Pharmacol.* **225,** 1-5.

Brzezinski P. and Gennis R. B. (2008) Cytochrome c oxidase: exciting progress and remaining mysteries. *J. Bioenerg. Biomembr.* **40,** 521-531.

Buck S. H., Murphy R. C. and Molinoff P. B. (1977) Normal occurrence of octopamine in central nervous system of rat. *Brain Res.* **122,** 281-297.

Burchett S. A. and Hicks T. P. (2006) The mysterious trace amines: protean neuromodulators of synaptic transmission in mammalian brain. *Prog. Neurobiol.* **79,** 223-246.

Burkhard P., Dominici P., Borri-Voltattorni C., Jansonius J. N. and Malashkevich V. N. (2001) Structural insight into Parkinson's disease treatment from drug-inhibited DOPA decarboxylase. *Nat. Struct. Biol.* **8,** 963-967.

Byrne G. I., Lehmann L. K. and Landry G. J. (1986) Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular Chlamydia psittaci replication in T24 cells. *Infect. Immun.* **53,** 347-351.

Calabrese V., Scapagnini G., Colombrita C., Ravagna A., Pennisi G., Giuffrida Stella A. M., Galli F. and Butterfield D. A. (2003) Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. *Amino Acids* **25,** 437-444.

Calabrese V., Mancuso C., Ravagna A., Perluigi M., Cini C., De Marco C., Butterfield D. A. and Stella A. M. (2007) In vivo induction of heat shock proteins in the substantia nigra following L-DOPA administration is associated with increased activity of

mitochondrial complex I and nitrosative stress in rats: regulation by glutathione redox state. *J. Neurochem.* **101,** 709-717.

Carafoli E. and Crompton M. (1978) The regulation of intracellular calcium by mitochondria. *Ann. N. Y. Acad. Sci.* **307,** 269-284.

Carlsson A., Lindqvist M. and Magnusson T. (1957) 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature* **180,** 1200.

Carlsson A., Lindqvist M., Magnusson T. and Waldeck B. (1958) On the presence of 3-hydroxytyramine in brain. *Science* **127,** 471.

Carlsson T., Bjorklund T. and Kirik D. (2007) Restoration of the striatal dopamine synthesis for Parkinson's disease: viral vector-mediated enzyme replacement strategy. *Curr. Gene Ther.* **7,** 109-120.

Caron M. G., Beaulieu M., Raymond V., Gagne B., Drouin J., Lefkowitz R. J. and Labrie F. (1978) Dopaminergic receptors in anterior-pituitary gland - correlation of [H-3]dihydroergocryptine binding with dopaminergic control of prolactin-release. *J. Biol. Chem.* **253,** 2244-2253.

Cartier E. A., Parra L. A., Baust T. B., Quiroz M., Salazar G., Faundez V., Egana L. and Torres G. E. (2010) A biochemical and functional protein complex involving dopamine synthesis and transport into synaptic vesicles. *J. Biol. Chem.* **285,** 1957-1966.

Caudle W. M., Richardson J. R., Wang M. Z., Taylor T. N., Guillot T. S., McCormack A. L., Colebrooke R. E., Di Monte D. A., Emson P. C. and Miller G. W. (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *J. Neurosci.* **27,** 8138-8148.

Ceccarelli.B, Hurlbut W. P. and Mauro A. (1973) Turnover of transmitter and synaptic vesicles at frog neuromuscular junction. *J. Cell Biol.* **57,** 499-524.

Cecchini G. (2003) Function and structure of complex II of the respiratory chain. *Annu. Rev. Biochem.* **72,** 77-109.

Ceravolo R., Piccini P., Bailey D. L., Jorga K. M., Bryson H. and Brooks D. J. (2002) F-18-dopa PET evidence that tolcapone acts as a central COMT inhibitor in Parkinson's disease. *Synapse* **43,** 201-207.

Chang Y. T., Mues G. and Hyland K. (1996) Alternative splicing in the coding region of human aromatic L-amino acid decarboxylase mRNA. *Neurosci. Lett.* **202,** 157-160.

Chang Y. T., Mues G., McPherson J. D., Bedell J. A., Marsh J. L. and Hyland K. (1998) Mutations in the human aromatic L-amino acid decarboxylase gene. *J. Inherit. Metab. Dis.* **21 (Suppl. 2),** 4.

Chang Y. T., Sharma R., Marsh J. L., McPherson J. D., Bedell J. A., Knust A., Brautigam C., Hoffmann G. F. and Hyland K. (2004) Levodopa-responsive aromatic L-amino acid decarboxylase deficiency. *Ann. Neurol.* **55,** 435-438.

Chao C. C., Hu S., Molitor T. W., Shaskan E. G. and Peterson P. K. (1992) Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J. Immunol.* **149,** 2736-2741.

Charteris A. and John R. (1975) An investigation of the assay of dopamine using trinitrobenzensulphonic acid. *Anal. Biochem.* **66,** 365-371.

Chen L., Ding Y., Cagniard B., Van Laar A. D., Mortimer A., Chi W., Hastings T. G., Kang U. J. and Zhuang X. (2008a) Unregulated cytosolic dopamine causes neurodegeneration associated with oxidative stress in mice. *J. Neurosci.* **28,** 425-433.

Chen P., Li A., Zhang M., He M., Chen Z., Wu X., Zhao C., Wang S. and Liang L. (2008b) Protective effects of a new metalloporphyrin on paraquat-induced oxidative stress and apoptosis in N27 cells. *Acta Biochim. Biophys. Sin. (Shanghai)* **40,** 125-132.

Chernyak B. V. and Bernardi P. (1996) The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites. *Eur. J. Biochem.* **238,** 623-630.

Chiarugi A., Dello S. P., Paccagnini A., Donnini S., Filippi S. and Moroni F. (2000) Combined inhibition of indoleamine 2,3-dioxygenase and nitric oxide synthase modulates neurotoxin release by interferon-gamma-activated macrophages. *J. Leukoc. Biol.* **68,** 260-266.

Chinta S. J. and Andersen J. K. (2006) Reversible inhibition of mitochondrial complex I activity following chronic dopaminergic glutathione depletion in vitro: implications for Parkinson's disease. *Free Radic. Biol. Med.* **41,** 1442-1448.

Cho S., Duchemin A. M., Neff N. H. and Hadjiconstantinou M. (1996) Modulation of tyrosine hydroxylase and aromatic L-amino acid decarboxylase after inhibiting monoamine oxidase-A. *Eur. J. Pharmacol.* **314,** 51-59.

Cho S., Duchemin A. M., Neff N. H. and Hadjiconstantinou M. (1999) Tyrosine hydroxylase, aromatic L-amino acid decarboxylase and dopamine metabolism after chronic treatment with dopaminergic drugs. *Brain Res.* **830,** 237-245.

Ciccarone V., Spengler B. A., Meyers M. B., Biedler J. L. and Ross R. A. (1989) Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. *Cancer Res.* **49,** 219-225.

Cidon S. and Sihra T. S. (1989) Characterization of a H^+-ATPase in rat-brain synaptic vesicles - coupling to L-glutamate transport. *J. Biol. Chem.* **264,** 8281-8288.

Citron B. A., Kaufman S., Milstien S., Naylor E. W., Greene C. L. and Davis M. D. (1993) Mutation in the 4a-carbinolamine dehydratase gene leads to mild hyperphenylalaninemia with defective cofactor metabolism. *Am. J. Hum. Genet.* **53,** 768-774.

Clayton P. T. (2006) B6-responsive disorders: a model of vitamin dependency. *J. Inherit. Metab. Dis.* **29,** 317-326.

Clayton R., Clark J. B. and Sharpe M. (2005) Cytochrome c release from rat brain mitochondria is proportional to the mitochondrial functional deficit: implications for apoptosis and neurodegenerative disease. *J. Neurochem.* **92,** 840-849.

Clement M. V., Long L. H., Ramalingam J. and Halliwell B. (2002) The cytotoxicity of dopamine may be an artefact of cell culture. *J. Neurochem.* **81,** 414-421.

Coburn S. P., Mahuren J. D., Schaltenbrand W. E., Wostmann B. S. and Madsen D. (1981) Effects of vitamin B6 deficiency and 4'-deoxypyridoxine on pyridoxal phosphate concentrations, pyridoxine kinase and other aspects of metabolism in the rat. *J. Nutr.* **111,** 391-398.

Cohen G., Farooqui R. and Kesler N. (1997) Parkinson disease: a new link between monoamine oxidase and mitochondrial electron flow. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4890-4894.

Colasanti M., Mollace V., Cundari E., Massoud R., Nistico G. and Lauro G. M. (1993) The generation of nitric oxide participates in gamma IFN-induced MHC class II antigen expression by cultured astrocytoma cells. *Int. J. Immunopharmacol.* **15,** 763-771.

Colpaert F. C., Koek W. and Lategan A. (1989) L-5-Hydroxytryptophan-induced flat body posture in the rat: antagonism by ritanserin and potentiation after 5,7-dihydroxytryptamine. *Eur. J. Pharmacol.* **169,** 175-178.

Connor T. J., Starr N., O'Sullivan J. B. and Harkin A. (2008) Induction of indolamine 2,3-dioxygenase and kynurenine 3-monooxygenase in rat brain following a systemic inflammatory challenge: a role for IFN-gamma? *Neurosci. Lett.* **441,** 29-34.

Cools R., Calder A. J., Lawrence A. D., Clark L., Bullmore E. and Robbins T. W. (2005) Individual differences in threat sensitivity predict serotonergic modulation of amygdala response to fearful faces. *Psychopharmacology (Berl).* **180,** 670-679.

Cools R., Roberts A. C. and Robbins T. W. (2008) Serotoninergic regulation of emotional and behavioural control processes. *Trends Cogn. Sci.* **12,** 31-40.

Corradin S. B., Mauel J., Donini S. D., Quattrocchi E. and Ricciardi-Castagnoli P. (1993) Inducible nitric oxide synthase activity of cloned murine microglial cells. *Glia* **7,** 255-262.

Crockett M. J., Clark L. and Robbins T. W. (2009) Reconciling the role of serotonin in behavioral inhibition and aversion: acute tryptophan depletion abolishes punishment-induced inhibition in humans. *J. Neurosci.* **29,** 11993-11999.

Crofts A. R. (2004) The cytochrome bc1 complex: function in the context of structure. *Annu. Rev. Physiol.* **66,** 689-733.

Cullen B. R. (2005) RNAi the natural way. *Nat. Genet.* **37,** 1163-1165.

Curtius H. C., Baerlocher K. and Vollmin J. A. (1972) Pathogenesis of phenylketonuria: inhibition of DOPA and catecholamine synthesis in patients with phenylketonuria. *Clin. Chim. Acta* **42,** 235-239.

d'Ischia M., Costantini C. and Prota G. (1993) Reaction of dopamine with malondialdehyde - a possible underlying event in Parkinsons disease. *Tetrahedron Lett.* **34,** 3921-3924.

d'Ischia M., Napolitano A. and Costantini C. (1995) Reaction of malondialdehyde with amine neurotransmitters: formation and oxidation chemistry of fluorescent 1,4-dihydropyridine adducts. *Tetrahedron* **51,** 9501-9508.

Dahlstrom A. and Fuxe K. (1964) Evidence for existence of monoamine-containing neurons in central nervous system .I. Demonstration of monoamines in cell bodies of brain stem neurons. *Acta Physiol. Scand.* **62,** 1-55.

Dai X. and Zhu B. T. (2010) Indoleamine 2,3-dioxygenase tissue distribution and cellular localization in mice: implications for its biological functions. *J. Histochem. Cytochem.* **58,** 17-28.

Daniels A. J. and Reinhard J. F., Jr. (1988) Energy-driven uptake of the neurotoxin 1-methyl-4-phenylpyridinium into chromaffin granules via the catecholamine transporter. *J. Biol. Chem.* **263,** 5034-5036.

Darchen F., Scherman D., Desnos C. and Henry J. P. (1988) Characteristics of the transport of the quaternary ammonium 1-methyl-4-phenylpyridinium by chromaffin granules. *Biochem. Pharmacol.* **37,** 4381-4387.

de Lau L. M., Koudstaal P. J., Witteman J. C., Hofman A. and Breteler M. M. (2006) Dietary folate, vitamin B12, and vitamin B6 and the risk of Parkinson disease. *Neurology* **67,** 315-318.

de Marchena O., Guarnieri M. and McKhann G. (1974) Glutathione peroxidase levels in brain. *J. Neurochem.* **22,** 773-776.

De Vries S., Albracht S. P., Berden J. A. and Slater E. C. (1982) The pathway of electrons through OH2:cytochrome c oxidoreductase studied by pre-steady -state kinetics. *Biochim. Biophys. Acta* **681,** 41-53.

Decker T. and Lohmann-Matthes M. L. (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods* **115,** 61-69.

Demerle-Pallardy C., Lonchampt M. O., Chabrier P. E. and Braquet P. (1993) Nitric oxide synthase induction in glial cells: effect on neuronal survival. *Life Sci.* **52,** 1883-1890.

Derkach V., Surprenant A. and North R. A. (1989) 5-HT_3 receptors are membrane ion channels. *Nature* **339,** 706-709.

Desole M. S., Miele M., Esposito G., Fresu L. G., Migheli R., Zangani D., Sircana S., Grella G. and Miele E. (1995) Neuronal antioxidant system and MPTP-induced oxidative stress in the striatum and brain stem of the rat. *Pharmacol. Biochem. Behav.* **51,** 581-592.

DeVeaugh-Geiss J. and Manion L. (1978) High-dose pyridoxine in tardive dyskinesia. *J. Clin. Psychiatry* **39,** 573-575.

Diaz-Hernandez J. I., Almeida A., Delgado-Esteban M., Fernandez E. and Bolanos J. P. (2005) Knockdown of glutamate-cysteine ligase by small hairpin RNA reveals that both catalytic and modulatory subunits are essential for the survival of primary neurons. *J. Biol. Chem.* **280,** 38992-39001.

Dimos J. T., Rodolfa K. T., Niakan K. K., Weisenthal L. M., Mitsumoto H., Chung W., Croft G. F., Saphier G., Leibel R., Goland R., Wichterle H., Henderson C. E. and Eggan K. (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321,** 1218-1221.

Donald L. J. (1982) A description of human aromatic alpha-keto acid reductase. *Ann. Hum. Genet.* **46,** 299-306.

Doroudchi M. M., Liauw J., Heaton K., Zhen Z. and Forsayeth J. R. (2005) Adeno-associated virus-mediated gene transfer of human aromatic L-amino acid decarboxylase protects mixed striatal primary cultures from L-DOPA toxicity. *J. Neurochem.* **93,** 634-640.

Dotsch J., Harmjanz A., Christiansen H., Hanze J., Lampert F. and Rascher W. (2000) Gene expression of neuronal nitric oxide synthase and adrenomedullin in human neuroblastoma using real-time PCR. *Int. J. Cancer* **88,** 172-175.

Dringen R., Kranich O. and Hamprecht B. (1997) The gamma-glutamyl transpeptidase inhibitor acivicin preserves glutathione released by astroglial cells in culture. *Neurochem. Res.* **22,** 727-733.

Dringen R., Pfeiffer B. and Hamprecht B. (1999) Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. *J. Neurosci.* **19,** 562-569.

Dringen R., Gutterer J. M., Gros C. and Hirrlinger J. (2001) Aminopeptidase N mediates the utilization of the GSH precursor CysGly by cultured neurons. *J. Neurosci. Res.* **66,** 1003-1008.

Dringen R. and Hirrlinger J. (2003) Glutathione pathways in the brain. *Biol. Chem.* **384,** 505-516.

Duchemin A. M., Berry M. D., Neff N. H. and Hadjiconstantinou M. (2000) Phosphorylation and activation of brain aromatic L-amino acid decarboxylase by cyclic AMP-dependent protein kinase. *J. Neurochem.* **75,** 725-731.

Duchemin A. M., Neff N. H. and Hadjiconstantinou M. (2010) Aromatic l-amino acid decarboxylase phosphorylation and activation by PKGIalpha *in vitro*. *J. Neurochem.*

Dunathan H. C. (1966) Conformation and reaction specificity in pyridoxal phosphate enzymes. *Proc. Natl. Acad. Sci. U. S. A.* **55,** 712-716.

Dunham E. T. and Glynn I. M. (1961) Adenosinetriphosphatase activity and active movements of alkali metal ions. *J. Physiol. (Lond).* **156,** 274-293.

Duvoisin R. C., Yahr M. D. and Cote L. D. (1969) Pyridoxine reversal of L-dopa effects in Parkinsonism. *Trans. Am. Neurol. Assoc.* **94,** 81-84.

Eastman C. L. and Guilarte T. R. (1989) Cytotoxicity of 3-hydroxykynurenine in a neuronal hybrid cell line. *Brain Res.* **495,** 225-231.

Eaton M. J., Gudehithlu K. P., Quach T., Silvia C. P., Hadjiconstantinou M. and Neff N. H. (1993) Distribution of aromatic L-amino acid decarboxylase messenger RNA in mouse brain by in situ hybridization histology. *J. Comp. Neurol.* **337,** 640-654.

Eberling J. L., Jagust W. J., Christine C. W., Starr P., Larson P., Bankiewicz K. S. and Aminoff M. J. (2008) Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology* **70,** 1980-1983.

Elstner M., Morris C. M., Heim K., Lichtner P., Bender A., Mehta D., Schulte C., Sharma M., Hudson G., Goldwurm S., Giovanetti A., Zeviani M., Burn D. J., McKeith I. G., Perry R. H., Jaros E., Kruger R., Wichmann H. E., Schreiber S., Campbell H., Wilson J. F., Wright A. F., Dunlop M., Pistis G., Toniolo D., Chinnery P. F., Gasser T., Klopstock T., Meitinger T., Prokisch H. and Turnbull D. M. (2009) Single-cell expression profiling of dopaminergic neurons combined with association analysis identifies pyridoxal kinase as Parkinson's disease gene. *Ann. Neurol.* **66,** 792-798.

Erickson J. D., Eiden L. E. and Hoffman B. J. (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10993-10997.

Evered D. F. (1971) L-dopa as a vitamin-B6 antagonist. *Lancet* **1,** 914.

Everett G. M. and Wiegand R. G. (1962) Central amines and behavioural states: a critique and new data, in *Proceedings of the 1st international pharmacology meeting*, pp. 85-92. Pergamon Press, Oxford UK.

Factor S. A. (2008) Current status of symptomatic medical therapy in Parkinson's disease. *Neurotherapeutics* **5,** 164-180.

Fahn S. (2005) Does levodopa slow or hasten the rate of progression of Parkinson's disease? *J. Neurol.* **252 Suppl 4,** IV37-IV42.

Fan M. M. and Raymond L. A. (2007) N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. *Prog. Neurobiol.* **81,** 272-293.

Farooqui A. A. and Horrocks L. A. (1998) Lipid peroxides in the free radical pathophysiology of brain diseases. *Cell Mol. Neurobiol.* **18,** 599-608.

Fatania H. R. and Dalziel K. (1980) Intracellular distribution of NADP-linked isocitrate dehydrogenase, fumarase and citrate synthase in bovine heart muscle. *Biochim. Biophys. Acta* **631,** 11-19.

Felix C. C. and Sealy R. C. (1981) Electron spin resonance characterization of radicals from 3,4-dihydroxyphenylalanine: semi-quinone anions and their metal chelates. *J. Am. Chem. Soc.* **103,** 2831-2836.

Fellman J. H., Vanbellinghen P. J., Jones R. T. and Koler R. D. (1969) Soluble and mitochondrial forms of tyrosine aminotransferase. Relationship to human tyrosinemia. *Biochemistry (Mosc).* **8,** 615-622.

Fellman J. H., Joyce J. R. and Strandholm J. J. (1971) Analysis in human plasma of 3-methoxytyrosine: a metabolite of DOPA. *Clin. Chim. Acta* **32,** 313-315.

Feng Z., Qin C., Chang Y. and Zhang J. T. (2006) Early melatonin supplementation alleviates oxidative stress in a transgenic mouse model of Alzheimer's disease. *Free Radic. Biol. Med.* **40,** 101-109.

Fitzgerald J. C., Ufer C., De Girolamo L. A., Kuhn H. and Billett E. E. (2007) Monoamine oxidase-A modulates apoptotic cell death induced by staurosporine in human neuroblastoma cells. *J. Neurochem.* **103,** 2189-2199.

Fiumara A., Brautigam C., Hyland K., Sharma R., Lagae L., Stoltenborg B., Hoffmann G. F., Jaeken J. and Wevers R. A. (2002) Aromatic L-amino acid decarboxylase deficiency with hyperdopaminuria. Clinical and laboratory findings in response to different therapies. *Neuropediatrics* **33,** 203-208.

Folling A. (1934) Uber ausscheidung von phenylbrenztraubensaure in den harn als stoffwechselanormalie in verbindung mit imbezillitat. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* **227,** 169-176.

Fornal C. A., Metzler C. W., Marrosu F., RibierodoValle L. E. and Jacobs B. L. (1996) A subgroup of dorsal raphe serotonergic neurons in the cat is strongly activated during oral-buccal movements. *Brain Res.* **716,** 123-133.

Forrest C. M., Mackay G. M., Stoy N., Spiden S. L., Taylor R., Stone T. W. and Darlington L. G. (2010) Blood levels of kynurenines, interleukin-23 and soluble human leucocyte antigen-G at different stages of Huntington's disease. *J. Neurochem.* **112,** 112-122.

Foster A. C., Collins J. F. and Schwarcz R. (1983) On the excitotoxic properties of quinolinic acid, 2,3-piperidine dicarboxylic acids and structurally related compounds. *Neuropharmacology* **22,** 1331-1342.

Foster S. J. and Perkins J. P. (1977) Glucocorticoids increase the responsiveness of cells in culture to prostaglandin E1. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4816-4820.

Frei B., Kim M. C. and Ames B. N. (1990) Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4879-4883.

Friedman P. A., Kappelma A. H. and Kaufman S. (1972) Partial purification and characterization of tryptophan hydroxylase from rabbit hindbrain. *J. Biol. Chem.* **247,** 4165-4173.

Froissard P., Monrocq H. and Duval D. (1997) Role of glutathione metabolism in the glutamate-induced programmed cell death of neuronal-like PC12 cells. *Eur. J. Pharmacol.* **326,** 93-99.

Fu L. H. and Toda N. (1979) Structure-activity relationship of 5-hydroxykynurenamine analogues in isolated dog cerebral arteries. *Jpn. J. Pharmacol.* **29,** 789-796.

Fujiwara M., Shibata M., Nomiyama Y., Sugimoto T., Hirata F., Tokuyama T., Senoh S. and Hayaishi O. (1979) Formation of 5-hydroxykynurenine and 5-hydroxykynurenamine from 5-hydroxytryptophan in rabbit small intestine. *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1145-1149.

Fuxe K. (1965) Evidence for existence of monoamine neurons in central nervous system .4. Distribution of monoamine terminals in central nervous system. *Acta Physiol. Scand.* **S 64,** 37.

Fuxe K., Corrodi H., Hokfelt T. and Jonsson G. (1970) Central monoamine neurons and pituitary-adrenal activity. *Prog. Brain Res.* **32,** 42-56.

Garthwaite G. and Garthwaite J. (1986) Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci. Lett.* **66,** 193-198.

Garthwaite G. and Garthwaite J. (1987) Quinolinate mimics neurotoxic actions of N-methyl-D-aspartate in rat cerebellar slices. *Neurosci. Lett.* **79,** 35-39.

Gegg M. E., Beltran B., Salas-Pino S., Bolanos J. P., Clark J. B., Moncada S. and Heales S. J. (2003) Differential effect of nitric oxide on glutathione metabolism and mitochondrial function in astrocytes and neurones: implications for neuroprotection/neurodegeneration? *J. Neurochem.* **86,** 228-237.

Gegg M. E., Clark J. B. and Heales S. J. (2005) Co-culture of neurones with glutathione deficient astrocytes leads to increased neuronal susceptibility to nitric oxide and increased glutamate-cysteine ligase activity. *Brain Res.* **1036,** 1-6.

Geppert M., Goda Y., Hammer R. E., Li C., Rosahl T. W., Stevens C. F. and Sudhof T. C. (1994) Synaptotagmin-I - a major Ca^{2+} sensor for transmitter release at a central synapse. *Cell* **79,** 717-727.

Gershten M. J., Disbrow J. K. and Ruth J. A. (1983) 3H-dopamine accumulation by rat brain synaptic vesicles in a membrane-impermeable medium. *Life Sci.* **33,** 323-329.

Gilman A. G. (1987) G-proteins - transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56,** 615-649.

Gimenez E., Lavado A., Giraldo P. and Montoliu L. (2003) Tyrosinase gene expression is not detected in mouse brain outside the retinal pigment epithelium cells. *Eur. J. Neurosci.* **18,** 2673-2676.

Giros B., Jaber M., Jones S. R., Wightman R. M. and Caron M. G. (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379,** 606-612.

Gluck M., Ehrhart J., Jayatilleke E. and Zeevalk G. D. (2002) Inhibition of brain mitochondrial respiration by dopamine: involvement of H_2O_2 and hydroxyl radicals but not glutathione-protein-mixed disulfides. *J. Neurochem.* **82,** 66-74.

Gluck M. R. and Zeevalk G. D. (2004) Inhibition of brain mitochondrial respiration by dopamine and its metabolites: implications for Parkinson's disease and catecholamine-associated diseases. *J. Neurochem.* **91,** 788-795.

Gomez-Santos C., Ambrosio S., Ventura F., Ferrer I. and Reiriz J. (2002) TGF-beta1 increases tyrosine hydroxylase expression by a mechanism blocked by BMP-2 in human neuroblastoma SH-SY5Y cells. *Brain Res.* **958,** 152-160.

Goodall M. (1951) Studies of adrenaline and noradrenaline in mammalian heart and suprarenals. *Acta Physiol Scand. Suppl* **24,** 7-51.

Goodwin B. L., Ruthven C. R., King G. S. and Sandler M. (1978) Metabolism of 3, 4-dihydroxyphenylalanine, its metabolites and analogues in vivo in the rat: urinary excretion pattern. *Xenobiotica* **8,** 629-651.

Goto H. and Inagaki M. (2007) Production of a site- and phosphorylation state-specific antibody. *Nat. Protoc.* **2,** 2574-2581.

Grahame-smith D. G. (1964) Tryptophan hydroxylation in brain. *Biochem. Biophys. Res. Commun.* **16,** 586-592.

Green H. and Sawyer J. L. (1966) Demonstration characterization and assay procedure of tryptophan hydroxylase in rat brain. *Anal. Biochem.* **15,** 53-64.

Greengard O. and Gordon M. (1963) Cofactor-mediated regulation of apoenzyme levels in animal tissues .1. Pyridoxine-induced rise of rat liver tyrosine transaminase level *in vivo*. *J. Biol. Chem.* **238,** 3708-3710.

Gross-Mesilaty S., Hargrove J. L. and Ciechanover A. (1997) Degradation of tyrosine aminotransferase (TAT) via the ubiquitin-proteasome pathway. *FEBS Lett.* **405,** 175-180.

Gu H., Wall S. C. and Rudnick G. (1994) Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J. Biol. Chem.* **269,** 7124-7130.

Guidetti P. and Schwarcz R. (1999) 3-Hydroxykynurenine potentiates quinolinate but not NMDA toxicity in the rat striatum. *Eur. J. Neurosci.* **11,** 3857-3863.

Guidetti P. and Schwarcz R. (2003) 3-Hydroxykynurenine and quinolinate: pathogenic synergism in early grade Huntington's disease? *Adv. Exp. Med. Biol.* **527,** 137-145.

Guilarte T. R., Wagner H. N., Jr. and Frost J. J. (1987) Effects of perinatal vitamin B6 deficiency on dopaminergic neurochemistry. *J. Neurochem.* **48,** 432-439.

Guillemin G. J., Williams K. R., Smith D. G., Smythe G. A., Croitoru-Lamoury J. and Brew B. J. (2003) Quinolinic acid in the pathogenesis of Alzheimer's disease. *Adv. Exp. Med. Biol.* **527,** 167-176.

Guillemin G. J., Brew B. J., Noonan C. E., Takikawa O. and Cullen K. M. (2005a) Indoleamine 2,3 dioxygenase and quinolinic acid immunoreactivity in Alzheimer's disease hippocampus. *Neuropathol. Appl. Neurobiol.* **31,** 395-404.

Guillemin G. J., Smythe G., Takikawa O. and Brew B. J. (2005b) Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia* **49,** 15-23.

Guillemin G. J., Kerr S. J. and Brew B. J. (2005c) Involvement of quinolinic acid in AIDS dementia complex. *Neurotox. Res.* **7,** 103-123.

Guillemin G. J., Cullen K. M., Lim C. K., Smythe G. A., Garner B., Kapoor V., Takikawa O. and Brew B. J. (2007) Characterization of the kynurenine pathway in human neurons. *J. Neurosci.* **27,** 12884-12892.

Guo S., Bezard E. and Zhao B. (2005) Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS-NO pathway. *Free Radic. Biol. Med.* **39,** 682-695.

Gutteridge J. M. (1994) Hydroxyl radicals, iron, oxidative stress, and neurodegeneration. *Ann. N. Y. Acad. Sci.* **738,** 201-213.

Guttman M., Leger G., Cedarbaum J. M., Reches A., Woodward W., Evans A., Diksic M. and Gjedde A. (1992) 3-O-methyldopa administration does not alter fluorodopa transport into the brain. *Ann. Neurol.* **31,** 638-643.

Gwaltney-Brant S. M., Albretsen J. C. and Khan S. A. (2000) 5-Hydroxytryptophan toxicosis in dogs: 21 cases (1989-1999). *J. Am. Vet. Med. Assoc.* **216,** 1937-1940.

Haavik J., Blau N. and Thony B. (2008) Mutations in human monoamine-related neurotransmitter pathway genes. *Hum. Mutat.* **29,** 891-902.

Haber S. N. (2002) The place of the dopamine neurons within the organization of the forebrain, in *Dopamine in the CNS,* (Di Chiara G., ed), pp. 43-62. Springer-Verlag, Berlin.

Haddad P. M. and Dursun S. M. (2008) Neurological complications of psychiatric drugs: clinical features and management. *Hum. Psychopharmacol.* **23 Suppl 1,** 15-26.

Hadjiconstantinou M., Rossetti Z., Silvia C., Krajnc D. and Neff N. H. (1988) Aromatic L-amino acid decarboxylase activity of the rat retina is modulated *in vivo* by environmental light. *J. Neurochem.* **51,** 1560-1564.

Hadjiconstantinou M., Wemlinger T. A., Sylvia C. P., Hubble J. P. and Neff N. H. (1993) Aromatic L-amino acid decarboxylase activity of mouse striatum is modulated via dopamine receptors. *J. Neurochem.* **60,** 2175-2180.

Hall A. S., Bryson S. E., Vaughan P. F., Ball S. G. and Balmforth A. J. (1993) Pharmacological characterization of the dopamine receptor coupled to cyclic AMP formation expressed by rat mesenteric artery vascular smooth muscle cells in culture. *Br. J. Pharmacol.* **110,** 681-686.

Halliwell B. and Gutteridge J. M. (1984) Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet* **1,** 1396-1397.

Halliwell B. (1992) Reactive oxygen species and the central nervous system. *J. Neurochem.* **59,** 1609-1623.

Halliwell B. (2006) Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* **97,** 1634-1658.

Han S. K., Mytilineou C. and Cohen G. (1996) L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. *J. Neurochem.* **66,** 501-510.

Haque M. E., Asanuma M., Higashi Y., Miyazaki I., Tanaka K. and Ogawa N. (2003) Apoptosis-inducing neurotoxicity of dopamine and its metabolites via reactive quinone generation in neuroblastoma cells. *Biochim. Biophys. Acta* **1619,** 39-52.

Hara H., Ohta M., Ohta K., Kuno S. and Adachi T. (2003) Increase of antioxidative potential by tert-butylhydroquinone protects against cell death associated with 6-hydroxydopamine-induced oxidative stress in neuroblastoma SH-SY5Y cells. *Brain Res. Mol. Brain Res.* **119,** 125-131.

Hardeland R., Reiter R. J., Poeggeler B. and Tan D. X. (1993) The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. *Neurosci. Biobehav. Rev.* **17,** 347-357.

Hargreaves I. P., Heales S. J. and Land J. M. (1999) Mitochondrial respiratory chain defects are not accompanied by an increase in the activities of lactate dehydrogenase or manganese superoxide dismutase in paediatric skeletal muscle biopsies. *J. Inherit. Metab. Dis.* **22,** 925-931.

Hargreaves I. P., Duncan A. J., Wu L., Agrawal A., Land J. M. and Heales S. J. (2007) Inhibition of mitochondrial complex IV leads to secondary loss complex II-III activity: implications for the pathogenesis and treatment of mitochondrial encephalomyopathies. *Mitochondrion.* **7,** 284-287.

Harrison W. W., Netsky M. G. and Brown M. D. (1968) Trace elements in human brain: copper, zinc, iron, and magnesium. *Clin. Chim. Acta* **21,** 55-60.

Hartig P. R. (1997) Molecular biology and transductional characteristics of 5-HT receptors, in *Serotoninergic neurons and 5-HT receptors in the CNS,* (Baumgarten H. G. and Gothert M., eds),Vol. 129, pp. 175-212. Springer-Verlag, Berlin.

Hassler R. (1938) Zur pathologie der paralysis agitans und des postenzephalitischen parkinsonismus. *J. Psychol. Neurol. (Lpz.)* **48,** 387-476.

Hayashi A., Suzuki H., Itoh K., Yamamoto M. and Sugiyama Y. (2003) Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochem. Biophys. Res. Commun.* **310,** 824-829.

Hayashi M., Yamaji Y., Kitajima W. and Saruta T. (1990) Aromatic L-amino acid decarboxylase activity along the rat nephron. *Am. J. Physiol.* **258,** F28-F33.

Hayes J. D., Flanagan J. U. and Jowsey I. R. (2005) Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **45,** 51-88.

Heales S. J., Bolanos J. P., Land J. M. and Clark J. B. (1994) Trolox protects mitochondrial complex IV from nitric oxide-mediated damage in astrocytes. *Brain Res.* **668,** 243-245.

Heales S. J., Davies S. E., Bates T. E. and Clark J. B. (1995) Depletion of brain glutathione is accompanied by impaired mitochondrial function and decreased N-acetyl aspartate concentration. *Neurochem. Res.* **20,** 31-38.

Hearing V. J. and Ekel T. M. (1976) Mammalian tyrosinase. A comparison of tyrosine hydroxylation and melanin formation. *Biochem. J.* **157,** 549-557.

Heckmann C. J., Gorassini M. A. and Bennett D. J. (2005) Persistent inward currents in motoneuron dendrites: implications for motor output. *Muscle Nerve* **31,** 135-156.

Hefti F., Melamed E., Bhawan J. and Wurtman R. J. (1981) Long-term administration of L-dopa does not damage dopaminergic-neurons in the mouse. *Neurology* **31,** 1194-1195.

Heisler L. K., Cowley M. A., Tecott L. H., Fan W., Low M. J., Smart J. L., Rubinstein M., Tatro J. B., Marcus J. N., Holstege H., Lee C. E., Cone R. D. and Elmquist J. K. (2002) Activation of central melanocortin pathways by fenfluramine. *Science* **297,** 609-611.

Heisler L. K., Jobst E. E., Sutton G. M., Zhou L. G., Borok E., Thornton-Jones Z., Liu H. Y., Zigman J. M., Balthasar N., Kishi T., Lee C. E., Aschkenasi C. J., Zhang C. Y., Yu J., Boss O., Mountjoy K. G., Clifton P. G., Lowell B. B., Friedman J. M., Horvath T., Butler A. A., Elmquist J. K. and Cowley M. A. (2006) Serotonin reciprocally regulates melanocortin neurons to modulate food intake. *Neuron* **51,** 239-249.

Herraiz T. and Galisteo J. (2004) Endogenous and dietary indoles: a class of antioxidants and radical scavengers in the ABTS assay. *Free Radic. Res.* **38,** 323-331.

Herrero-Mendez A., Almeida A., Fernandez E., Maestre C., Moncada S. and Bolanos J. P. (2009) The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat. Cell Biol.* **11,** 747-752.

Hess S. M., Redfield B. G. and Udenfriend S. (1959) The effect of monoamine oxidase inhibitors and tryptophan on the tryptamine content of animal tissues and urine. *J. Pharmacol. Exp. Ther.* **127,** 178-181.

Hess S. M., Ozaki M., Udenfriend S. and Connamac R. H. (1961) Effects of alpha-methyldopa and alpha-methyl-meta-tyrosine on metabolism of norepinephrine and serotonin *in vivo*. *J. Pharmacol. Exp. Ther.* **134,** 129-138.

Heuser J. E. and Reese T. S. (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at frog neuromuscular junction. *J. Cell Biol.* **57,** 315-344.

Heyes M. P., Saito K., Major E. O., Milstien S., Markey S. P. and Vickers J. H. (1993) A mechanism of quinolinic acid formation by brain in inflammatory neurological disease. Attenuation of synthesis from L-tryptophan by 6-chlorotryptophan and 4-chloro-3-hydroxyanthranilate. *Brain* **116 (Pt 6),** 1425-1450.

Hillarp N. A., Fuxe K. and Dahlstrom A. (1966) Demonstration and mapping of central neurons containing dopamine, noradrenaline, and 5-hydroxytryptamine and their reactions to psychopharmaca. *Pharmacol. Rev.* **18,** 727-741.

Hiller K., Metallo C. M., Kelleher J. K. and Stephanopoulos G. (2010) Nontargeted elucidation of metabolic pathways using stable-isotope tracers and mass spectrometry. *Anal. Chem.* **82,** 6621-6628.

Hirata F. and Hayaishi O. (1972) New degradative routes of 5-hydroxytryptophan and serotonin by intestinal tryptophan 2,3-dioxygenase. *Biochem. Biophys. Res. Commun.* **47,** 1112-1119.

Hirrlinger J., Schulz J. B. and Dringen R. (2002) Effects of dopamine on the glutathione metabolism of cultured astroglial cells: implications for Parkinson's disease. *J. Neurochem.* **82,** 458-467.

Hirst J. (2010) Towards the molecular mechanism of respiratory complex I. *Biochem. J.* **425,** 327-339.

Hoffman B. J., Mezey E. and Brownstein M. J. (1991) Cloning of a serotonin transporter affected by antidepressants. *Science* **254,** 579-580.

Hoffmann G. F., Assmann B., Brautigam C., Dionisi-Vici C., Haussler M., de Klerk J. B. C., Naumann M., Steenbergen-Spanjers G. C. H., Strassburg H. M. and Wevers R. A.

(2003) Tyrosine hydroxylase deficiency causes progressive encephalopathy and dopa-nonresponsive dystonia. *Ann. Neurol.* **54,** S56-S65.

Hoffmann G. F., Schmitt B., Windfuhr M., Wagner N., Strehl H., Bagci S., Franz A. R., Mills P. B., Clayton P. T., Baumgartner M. R., Steinmann B., Bast T., Wolf N. I. and Zschocke J. (2007) Pyridoxal 5'-phosphate may be curative in early-onset epileptic encephalopathy. *J. Inherit. Metab. Dis.* **30,** 96-99.

Hokfelt T., Fuxe K. and Goldstein M. (1973) Immunohistochemical localization of aromatic L-amino acid decarboxylase (dopa decarboxylase) in central dopamine and 5-hydroxytryptamine nerve cell bodies of rat. *Brain Res.* **53,** 175-180.

Holtz P., Heise R. and Ludtke K. (1938) Fermentativer abbau von l-dioxyphenylalanin (dopa) durch Niere. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **191,** 87-118.

Holtz P. (1939) Dopadecarboxylase. *Naturwissenschaften* **43,** 724-725.

Hornung J. P. (2003) The human raphe nuclei and the serotonergic system. *J. Chem. Neuroanat.* **26,** 331-343.

Hornykiewicz O. (1958) The action of dopamine on the arterial blood pressure of the guinea-pig. *Br. J. Pharmacol. Chemother.* **13,** 91-94.

Hornykiewicz O. (1963) Die topische lokalisation und verhalten von noradrenalin und dopamin (3-hydroxytyramin) in der substantia nigra des normalen und parkinsonkranken menschen. *Wiener Klin Wochenschr* **75,** 309-312.

Hornykiewicz O. (1966) Dopamine (3-hydroxytyramine) and brain function. *Pharmacol. Rev.* **18,** 925-964.

Hou J. G., Cohen G. and Mytilineou C. (1997) Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. *J. Neurochem.* **69,** 76-83.

Hsieh H. J., Lin S. H. and Liu H. M. (2005) Visualisation of impaired dopamine biosynthesis in a case of aromatic L-amino acid decarboxylase deficiency by co-registered F-18-FDOPA PET and magnetic resonance imaging. *Eur. J. Nucl. Med. Mol. Imaging* **32,** 517.

Huq M. D. M., Tsai N. P., Lin Y. P., Higgins L. and Wei L. N. (2007) Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. *Nat. Chem. Biol.* **3,** 161-165.

Hurwitz J. (1955) Enzymatic phosphorylation of vitamin B6 analogues and their effect on tyrosine decarboxylase. *J. Biol. Chem.* **217,** 513-525.

Hwu P., Du M. X., Lapointe R., Do M., Taylor M. W. and Young H. A. (2000) Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J. Immunol.* **164,** 3596-3599.

Hyland K. and Clayton P. T. (1990) Aromatic amino acid decarboxylase deficiency in twins. *J. Inherit. Metab. Dis.* **13,** 301-304.

Hyland K., Surtees R. A. H., Rodeck C. and Clayton P. T. (1992) Aromatic L-amino acid decarboxylase deficiency: clinical features, diagnosis, and treatment of a new inborn error of neurotransmitter amine synthesis. *Neurology* **42,** 1980-1988.

Hyland K. and Clayton P. T. (1992) Aromatic L-amino acid decarboxylase deficiency - diagnostic methodology. *Clin. Chem.* **38,** 2405-2410.

Hyland K. (1999) Presentation, diagnosis, and treatment of the disorders of monoamine neurotransmitter metabolism. *Semin. Perinatol.* **23,** 194-203.

Hyland K. (2007) Inherited disorders affecting dopamine and serotonin: Critical neurotransmitters derived from aromatic amino acids. *J. Nutr.* **137,** 1568S-1572S.

Hyland K. (2008) Clinical utility of monoamine neurotransmitter metabolite analysis in cerebrospinal fluid. *Clin. Chem.* **54,** 633-641.

Ichinose H., Kurosawa Y., Titani K., Fujita K. and Nagatsu T. (1989) Isolation and characterization of a cDNA clone encoding human aromatic L-amino acid decarboxylase. *Biochem. Biophys. Res. Commun.* **164,** 1024-1030.

Ichinose H., Sumi-Ichinose C., Ohye T., Hagino Y., Fujita K. and Nagatsu T. (1992) Tissue-specific alternative splicing of the first exon generates two types of mRNAs in human aromatic L-amino acid decarboxylase. *Biochemistry (Mosc).* **31,** 11546-11550.

Ikeda M., Tsuji H., Nakamura S., Ichiyama A., Nishizuka Y. and Hayaishi O. (1965) Studies on the biosynthesis of nicotinamide adenine dinucleotide II. a role of picolinic carboxylase in the biosynthesis of nicotinamide adenine dinucleotide from tryptophan in mammals. *J. Biol. Chem.* **240,** 1395-1401.

Ikemoto K. (2007) Neuropsychiatric diseases and monoamine neuronal systems. *Psychiatry Clin. Neurosci.* **61,** S22.

Iredale P. A., Terwilliger R., Widnell K. L., Nestler E. J. and Duman R. S. (1996) Differential regulation of corticotropin-releasing factor1 receptor expression by stress and agonist treatments in brain and cultured cells. *Mol. Pharmacol.* **50,** 1103-1110.

Ishii S., Mizuguchi H., Nishino J., Hayashi H. and Kagamiyama H. (1996) Functionally important residues of aromatic L-amino acid decarboxylase probed by sequence alignment and site-directed mutagenesis. *J. Biochem. (Tokyo).* **120,** 369-376.

Ishii S., Hayashi H., Okamoto A. and Kagamiyama H. (1998) Aromatic L-amino acid decarboxylase: Conformational change in the flexible region around Arg334 is required during the transaldimination process. *Protein Sci.* **7,** 1802-1810.

Ito S., Nakayama T., Ide S., Ito Y., Oguni H., Goto Y. I. and Osawa M. (2008) Aromatic l-amino acid decarboxylase deficiency associated with epilepsy mimicking non-epileptic involuntary movements. *Dev. Med. Child Neurol.*

Itoh K., Chiba T., Takahashi S., Ishii T., Igarashi K., Katoh Y., Oyake T., Hayashi N., Satoh K., Hatayama I., Yamamoto M. and Nabeshima Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236,** 313-322.

Itoh K., Wakabayashi N., Katoh Y., Ishii T., Igarashi K., Engel J. D. and Yamamoto M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13,** 76-86.

Itoh K., Wakabayashi N., Katoh Y., Ishii T., O'Connor T. and Yamamoto M. (2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* **8,** 379-391.

Iwasaki Y., Kato Y., Ohgo S., Abe H., Imura H., Hirata F., Senoh S., Tokuyama T. and Hayaishi O. (1978) Effects of indoleamines and their newly identified metabolites on prolactin release in rats. *Endocrinology* **103,** 254-258.

Izumi T., Iwamoto N., Kitaichi Y., Kato A., Inoue T. and Koyama T. (2006) Effects of co-administration of a selective serotonin reuptake inhibitor and monoamine oxidase inhibitors on 5-HT-related behavior in rats. *Eur. J. Pharmacol.* **532,** 258-264.

Jacobs B. L. and Fornal C. A. (1997a) Physiology and pharmacology of brain serotoninergic neurons, in *Serotoninergic neurons and 5-HT receptors in the CNS,* (Baumgarten H. G. and Gothert M., eds),Vol. 129, pp. 91-116. Spirnger-Verlag, Berlin.

Jacobs B. L. and Fornal C. A. (1997b) Serotonin and motor activity. *Curr. Opin. Neurobiol.* **7,** 820-825.

Jaeger C. B., Teitelman G., Joh T. H., Albert V. R., Park D. H. and Reis D. J. (1983) Some neurons of the rat central nervous system contain aromatic L-amino acid decarboxylase but not monoamines. *Science* **219,** 1233-1235.

Jaeger C. B., Ruggiero D. A., Albert V. R., Park D. H., Joh T. H. and Reis D. J. (1984) Aromatic L-amino acid decarboxylase in the rat brain: immunocytochemical localization in neurons of the brain stem. *Neuroscience* **11,** 691-713.

James A. M., Smith R. A. and Murphy M. P. (2004) Antioxidant and prooxidant properties of mitochondrial Coenzyme Q. *Arch. Biochem. Biophys.* **423,** 47-56.

Janssens N., Janicot M., Perera T. and Bakker A. (2004) Housekeeping genes as internal standards in cancer research. *Mol. Diagn.* **8,** 107-113.

Jebai F., Hanoun N., Hamon M., Thibault J., Peltre G., Gros F. and Krieger M. (1997) Expression, purification, and characterization of rat aromatic L-amino acid decarboxylase in Escherichia coli. *Protein Expr. Purif.* **11,** 185-194.

Jeon M. Y., Lee W. Y., Kang H. Y. and Chung E. J. (2007) The effects of L-3,4-dihydroxyphenylalanine and dopamine agonists on dopamine neurons in the progressive hemiparkinsonian rat models. *Neurol. Res.* **29,** 289-295.

Jia Z., Zhu H., Misra B. R., Li Y. and Misra H. P. (2008a) Dopamine as a potent inducer of cellular glutathione and NAD(P)H:quinone oxidoreductase 1 in PC12 neuronal cells: a potential adaptive mechanism for dopaminergic neuroprotection. *Neurochem. Res.* **33,** 2197-2205.

Jia Z., Zhu H., Misra H. P. and Li Y. (2008b) Potent induction of total cellular GSH and NQO1 as well as mitochondrial GSH by 3H-1,2-dithiole-3-thione in SH-SY5Y neuroblastoma cells and primary human neurons: protection against neurocytotoxicity elicited by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or hydrogen peroxide. *Brain Res.* **1197,** 159-169.

Jia Z., Hallur S., Zhu H., Li Y. and Misra H. P. (2008c) Potent upregulation of glutathione and NAD(P)H:quinone oxidoreductase 1 by alpha-lipoic acid in human neuroblastoma SH-SY5Y cells: protection against neurotoxicant-elicited cytotoxicity. *Neurochem. Res.* **33,** 790-800.

Jocelyn P. C. and Dickson J. (1980) Glutathione and the mitochondrial reduction of hydroperoxides. *Biochim. Biophys. Acta* **590,** 1-12.

Jones R. S. G. and Boulton A. A. (1980) Tryptamine and 5-hydroxytryptamine - actions and interactions on cortical neurons in the rat. *Life Sci.* **27,** 1849-1856.

Jonnakuty C. and Gragnoli C. (2008) What do we know about serotonin? *J. Cell. Physiol.* **217,** 301-306.

Kafi S. and Gaillard J. M. (1976) Brain dopamine receptors and sleep in rat: effects of stimulation and blockade. *Eur. J. Pharmacol.* **38,** 357-364.

Kalfon L., Youdim M. B. and Mandel S. A. (2007) Green tea polyphenol (-) - epigallocatechin-3-gallate promotes the rapid protein kinase C- and proteasome-mediated degradation of Bad: implications for neuroprotection. *J. Neurochem.* **100,** 992-1002.

Kalinina E. V., Chernov N. N. and Saprin A. N. (2008) Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry (Mosc.)* **73,** 1493-1510.

Kaspar J. W., Niture S. K. and Jaiswal A. K. (2009) Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic. Biol. Med.* **47,** 1304-1309.

Kaufman S. (1959) Studies on the mechanism of the enzymatic conversion of phenylalanine to tyrosine. *J. Biol. Chem.* **234,** 2677-2682.

Kaufman S., Holtzman N. A., Milstien S., Butler I. J. and Krumholz A. (1975) Phenylketonuria due to a deficiency of dihydropteridine reductase. *N. Engl. J. Med.* **293,** 785-790.

Kawasaki Y., Hayashi H., Hatakeyama K. and Kagamiyama H. (1992) Evaluation of the holoenzyme content of aromatic L-amino acid decarboxylase in brain and liver tissues. *Biochem. Biophys. Res. Commun.* **186,** 1242-1248.

Kebabian J. W. and Calne D. B. (1979) Multiple receptors for dopamine. *Nature* **277,** 93-96.

Kido R., Noguchi T. and Matsumura Y. (1967a) The isolation and identification of 5-hydroxy-L-kynurenine from the urine of hens. *Biochim. Biophys. Acta* **141,** 270-275.

Kido R., Noguchi T., Tsuji T. and Matsumura Y. (1967b) The identification of 4, 6-quinolinediol and 4,8-quinolinediol in the urine of hens. *Biochim. Biophys. Acta* **136,** 131-135.

Kilbourn R. G. and Belloni P. (1990) Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J. Natl. Cancer Inst.* **82,** 772-776.

Kilty J. E., Lorang D. and Amara S. G. (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* **254,** 578-579.

King T. E. (1967) Preparation of succinate cytochrome c reductase and cytochrome b-cl particle and reconstitution of succinate cytochrome c reductase. *Meth. Enzymol.* **10,** 217-235.

Kitahama K., Denoyer M., Raynaud B., Borri-Voltattorni C., Weber M. and Jouvet M. (1990) Aromatic L-amino acid decarboxylase-immunohistochemistry in the cat lower brainstem and midbrain. *J. Comp Neurol.* **302,** 935-953.

Kitahama K., Ikemoto K., Jouvet A., Araneda S., Nagatsu I., Raynaud B., Nishimura A., Nishi K. and Niwa S. (2009) Aromatic L-amino acid decarboxylase-immunoreactive structures in human midbrain, pons, and medulla. *J. Chem. Neuroanat.* **38,** 130-140.

Kitamura S., Shimizu T. and Ishihara Y. (1979) Effects of 5-hydroxykynurenamine, a metabolite of 5-hydroxytryptamine, on guinea-pig isolated trachea. *J. Pharm. Pharmacol.* **31,** 45-46.

Klawans H. L., Ringel S. P. and Shenker D. M. (1971) Failure of vitamin B6 to reverse the L-dopa effect in patients on a dopa decarboxylase inhibitor. *J. Neurol. Neurosurg. Psychiatry* **34,** 682-686.

Knoll J., Miklya N., Knoll B., Marko R. and Racz D. (1996) Phenylethylamine and tyramine are mixed-acting sympathomimetic amines in the brain. *Life Sci.* **58,** 2101-2114.

Knoth J., Handloser K. and Njus D. (1980) Electrogenic epinephrine transport in chromaffin granule ghosts. *Biochemistry (Mosc).* **19,** 2938-2942.

Knox W. E. and Mehler A. H. (1950) The conversion of tryptophan to kynurenine in liver. I. The coupled tryptophan peroxidase-oxidase system forming formylkynurenine. *J. Biol. Chem.* **187,** 419-430.

Koella W. P., Feldstein A. and Czicman J. S. (1968) Effect of para-chlorophenylalanine on sleep of cats. *Electroencephalogr. Clin. Neurophysiol.* **25,** 481-490.

Korenke G. C., Christen H. J., Hyland K., Hunneman D. H. and Hanefeld F. (1997) Aromatic L-amino acid decarboxylase deficiency: an extrapyramidal movement disorder with oculogyric crises. *Eur. J. Paediatr. Neurol.* **1,** 67-71.

Koshimura K., Tanaka J., Murakami Y. and Kato Y. (2000) Effects of dopamine and L-DOPA on survival of PC12 cells. *J. Neurosci. Res.* **62,** 112-119.

Kranich O., Hamprecht B. and Dringen R. (1996) Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and astroglial cells derived from rat brain. *Neurosci. Lett.* **219,** 211-214.

Kranich O., Dringen R., Sandberg M. and Hamprecht B. (1998) Utilization of cysteine and cysteine precursors for the synthesis of glutathione in astroglial cultures: preference for cystine. *Glia* **22,** 11-18.

Kreutzberg G. W. (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **19,** 312-318.

Krieger M., Coge F., Gros F. and Thibault J. (1991) Different mRNAs code for dopa decarboxylase in tissues of neuronal and nonneuronal origin. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2161-2165.

Kudin A. P., Debska-Vielhaber G. and Kunz W. S. (2005) Characterization of superoxide production sites in isolated rat brain and skeletal muscle mitochondria. *Biomed. Pharmacother.* **59,** 163-168.

Kurian M. A., Zhen J., Cheng S. Y., Li Y., Mordekar S. R., Jardine P., Morgan N. V., Meyer E., Tee L., Pasha S., Wassmer E., Heales S. J. R., Gissen P., Reith M. E. A. and Maher E. R. (2009) Homozygous loss-of-function mutations in the gene encoding the dopamine transporter are associated with infantile parkinsonism-dystonia. *J. Clin. Invest.* **119,** 1595-1603.

Kvernmo T., Hartter S. and Burger E. (2006) A review of the receptor-binding and pharmacokinetic properties of dopamine agonists. *Clin. Ther.* **28,** 1065-1078.

Kwidzinski E., Bunse J., Aktas O., Richter D., Mutlu L., Zipp F., Nitsch R. and Bechmann I. (2005) Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. *FASEB J.* **19,** 1347-1349.

Lai C. T. and Yu P. H. (1997) Dopamine- and L-beta-3,4-dihydroxyphenylalanine hydrochloride (L-Dopa)-induced cytotoxicity towards catecholaminergic neuroblastoma

SH-SY5Y cells. Effects of oxidative stress and antioxidative factors. *Biochem. Pharmacol.* **53,** 363-372.

Lambert A. J. and Brand M. D. (2004) Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J. Biol. Chem.* **279,** 39414-39420.

Lane R. and Baldwin D. (1997) Selective serotonin reuptake inhibitor-induced serotonin syndrome: review. *J. Clin. Psychopharmacol.* **17,** 208-221.

Lauweryns J. M. and Van Ranst L. (1988) Immunocytochemical localization of aromatic L-amino acid decarboxylase in human, rat, and mouse bronchopulmonary and gastrointestinal endocrine cells. *J. Histochem. Cytochem.* **36,** 1181-1186.

Lee d. Y., Lee K. S., Lee H. J., Noh Y. H., Kim d. H., Lee J. Y., Cho S. H., Yoon O. J., Lee W. B., Kim K. Y., Chung Y. H. and Kim S. S. (2008c) Kynurenic acid attenuates MPP(+)-induced dopaminergic neuronal cell death via a bax-mediated mitochondrial pathway. *Eur. J. Cell Biol.* **87,** 389-397.

Lee E. S., Chen H., King J. and Charlton C. (2008d) The role of 3-O-methyldopa in the side effects of L-dopa. *Neurochem. Res.* **33,** 401-411.

Lee H. F., Tsai C. R., Chi C. S., Chang T. M. and Lee H. J. (2008a) Aromatic l-amino acid decarboxylase deficiency in Taiwan. *Eur. J. Paediatr. Neurol.* **13,** 135-140.

Lee N. C., Hwu W. L., Chien Y. H., Lee W. Y., Huang A. C. and Yang C. R. (2008b) Prevalence of the IVS6+4 A>T mutation in taiwanese patients with aromatic amino acid decarboxylase deficiency. *J. Inherit. Metab. Dis.* **31 (Suppl. 1),** 136.

Lee W. T., Weng W. C., Peng S. F. and Tzen K. Y. (2009) Neuroimaging findings in children with paediatric neurotransmitter diseases. *J. Inherit. Metab. Dis.* **32,** 361-370.

Lehmann A. (1987) Pharmacological protection against the toxicity of N-methyl-D-aspartate in immature rat cerebellar slices. *Neuropharmacology* **26,** 1751-1761.

Leonard B. E. (2007) Inflammation, depression and dementia: are they connected? *Neurochem. Res.* **32,** 1749-1756.

Lerner V., Kaptsan A., Miodownik C. and Kotler M. (1999) Vitamin B6 in treatment of tardive dyskinesia: a preliminary case series study. *Clin. Neuropharmacol.* **22,** 241-243.

Lerner V., Miodownik C., Kaptsan A., Bersudsky Y., Libov I., Sela B. A. and Witztum E. (2007) Vitamin B6 treatment for tardive dyskinesia: a randomized, double-blind, placebo-controlled, crossover study. *J. Clin. Psychiatry* **68,** 1648-1654.

Levin E. Y., Levenberg B. and Kaufman S. (1960) Enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J. Biol. Chem.* **235,** 2080-2086.

Levitt P., Pintar J. E. and Breakefield X. O. (1982) Immunocytochemical demonstration of monoamine oxidase-B in brain astrocytes and serotonergic neurons. *Proc. Natl. Acad. Sci. U. S. A.* **79,** 6385-6389.

LeWitt P. A. and Nyholm D. (2004) New developments in levodopa therapy. *Neurology* **62,** S9-16.

Li X. M., Juorio A. V. and Boulton A. A. (1993) NSD-1015 alters the gene expression of aromatic L-amino acid decarboxylase in rat PC12 pheochromocytoma cells. *Neurochem. Res.* **18,** 915-919.

Lineweaver H. and Burk D. (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56,** 658-666.

Littlejohn T. K., Takikawa O., Skylas D., Jamie J. F., Walker M. J. and Truscott R. J. (2000) Expression and purification of recombinant human indoleamine 2, 3-dioxygenase. *Protein Expr. Purif.* **19,** 22-29.

Liu Y. J., Peter D., Roghani A., Schuldiner S., Prive G. G., Eisenberg D., Brecha N. and Edwards R. H. (1992) A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter. *Cell* **70,** 539-551.

Longo N. (2009) Disorders of biopterin metabolism. *J. Inherit. Metab. Dis.* **32,** 333-342.

Lovenberg W., Weissbach H. and Udenfriend S. (1962) Aromatic L-amino acid decarboxylase. *J. Biol. Chem.* **237,** 89-93.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Luciana M., Depue R. A., Arbisi P. and Leon A. (1992) Facilitation of working memory in humans by a D2-dopamine receptor agonist. *J. Cogn. Neurosci.* **4,** 58-68.

Luciana M. and Collins P. F. (1997) Dopaminergic modulation of working memory for spatial but not object cues in normal humans. *J. Cogn. Neurosci.* **9,** 330-347.

Lucki I. (1998) The spectrum of behaviors influenced by serotonin. *Biol. Psychiatry* **44,** 151-162.

Ludecke B., Knappskog P. M., Clayton P. T., Surtees R. A. H., Clelland J. D., Heales S. J. R., Brand M. P., Bartholome K. and Flatmark T. (1996) Recessively inherited L-

DOPA-responsive parkinsonism in infancy caused by a point mutation (L205P) in the tyrosine hydroxylase gene. *Hum. Mol. Genet.* **5,** 1023-1028.

Lumeng L., Brashear R. E. and Li T. K. (1974) Pyridoxal 5'-phosphate in plasma: source, protein-binding, and cellular transport. *J. Lab Clin. Med.* **84,** 334-343.

Lumeng L., Lui A. and Li T. K. (1980) Plasma content of B6 vitamers and its relationship to hepatic vitamin B6 metabolism. *J. Clin. Invest* **66,** 688-695.

Macintyre E. H., Ponten J. and Vatter A. E. (1972) The ultrastructure of human and murine astrocytes and of human fibroblasts in culture. *Acta Pathol. Microbiol. Scand. A* **80,** 267-283.

Mackowiak E. D., Hare T. A. and Vogel W. H. (1972) Measurement of aromatic L-amino acid decarboxylase--a technical comment. *Biochem. Med.* **6,** 562-567.

Maeda T., Tanaka M., Tanaka K. and Shindo H. (1978) Metabolic pathway of L-3-methoxy-4-hydroxyphenyl-alanine (3-O-methyl dopa) participation of tyrosine aminotransferase, aromatic α-ketoacid reductase and lactate dehydrogenase. *J. Pharmacobiodyn.* **1,** 288-300.

Magyar K., Palfi M., Jenei V. and Szoko E. (2006) Deprenyl: from chemical synthesis to neuroprotection. *J. Neural Transm. Suppl.* **71,** 143-156.

Makar T. K., Nedergaard M., Preuss A., Gelbard A. S., Perumal A. S. and Cooper A. J. (1994) Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. *J. Neurochem.* **62,** 45-53.

Maller A., Hyland K., Milstien S., Biaggioni I. and Butler I. J. (1997) Aromatic L-amino acid decarboxylase deficiency: Clinical features, diagnosis, and treatment of a second family. *J. Child Neurol.* **12,** 349-354.

Mandeles S. and Bloch K. (1955) Enzymatic synthesis of gamma-glutamylcysteine. *J. Biol. Chem.* **214,** 639-646.

Manegold C., Hoffmann G. F., Degen I., Ikonomidou H., Knust A., Laass M. W., Pritsch M., Wilichowski E. and Horster F. (2009) Aromatic L-amino acid decarboxylase deficiency: clinical features, drug therapy and follow-up. *J. Inherit. Metab. Dis.* **32,** 371-380.

Mappouras D. G., Stiakakis J. and Fragoulis E. G. (1990) Purification and characterization of L-dopa decarboxylase from human kidney. *Mol. Cell Biochem.* **94,** 147-156.

Maring J. A., Deitrich R. A. and Little R. (1985) Partial purification and properties of human brain aldehyde dehydrogenases. *J. Neurochem.* **45,** 1903-1910.

Maronde E. and Stehle J. H. (2007) The mammalian pineal gland: known facts, unknown facets. *Trends Endocrinol. Metab* **18,** 142-149.

Marsden C. A. and Joseph M. H. (1986) Biogenic amines, in *HPLC of small molecules. A practical approach,* (Lim C. K., ed), pp. 29-47. IRL Press, Oxford, UK.

Martensson J., Lai J. C. and Meister A. (1990) High-affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7185-7189.

Martin D. M., Carlson R. O. and Feldman E. L. (1993) Interferon-gamma inhibits DNA synthesis and insulin-like growth factor-II expression in human neuroblastoma cells. *J. Neurosci. Res.* **34,** 489-501.

Martin H. L. and Teismann P. (2009) Glutathione--a review on its role and significance in Parkinson's disease. *FASEB J.* **23,** 3263-3272.

Maruyama W., Akao Y., Carrillo M. C., Kitani K., Youdium M. B. and Naoi M. (2002) Neuroprotection by propargylamines in Parkinson's disease: suppression of apoptosis and induction of prosurvival genes. *Neurotoxicol. Teratol.* **24,** 675-682.

Mash D. C., Staley J. K., Izenwasser S., Basile M. and Ruttenber A. J. (2000) Serotonin transporters upregulate with chronic cocaine use. *J. Chem. Neuroanat.* **20,** 271-280.

Matsuda N., Hayashi H., Miyatake S., Kuroiwa T. and Kagamiyama H. (2004) Instability of the Apo form of aromatic L-amino acid decarboxylase in vivo and in vitro: Implications for the involvement of the flexible loop that covers the active site. *J. Biochem. (Tokyo).* **135,** 33-42.

Mavelli I., Rigo A., Federico R., Ciriolo M. R. and Rotilio G. (1982) Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. *Biochem. J.* **204,** 535-540.

McCormick D. B. and Snell E. E. (1959) Pyridoxal kinase of human brain and its inhibition by hydrazine derivatives. *Proc. Natl. Acad. Sci. U. S. A.* **45,** 1371-1379.

McCormick D. B., Gregory M. E. and Snell E. E. (1961) Pyridoxal phosphokinases. I. assay, distribution, I. assay, distribution, purification, and properties. *J. Biol. Chem.* **236,** 2076-2084.

McEwen C. M. J. (1965) Human plasma monoamine oxidase. 1. Purification and identification. *J. Biol. Chem.* **240,** 2003-2010.

McKean C. M., Boggs D. E. and Peterson N. A. (1968) The influence of high phenylalanine and tyrosine on the concentrations of essential amino acids in brain. *J. Neurochem.* **15,** 235-241.

McKean C. M. (1972) The effects of high phenylalanine concentrations on serotonin and catecholamine metabolism in the human brain. *Brain Res.* **47,** 469-476.

McLaughlin D., Tsirimonaki E., Vallianatos G., Sakellaridis N., Chatzistamatiou T., Stavropoulos-Gioka C., Tsezou A., Messinis I. and Mangoura D. (2006) Stable expression of a neuronal dopaminergic progenitor phenotype in cell lines derived from human amniotic fluid cells. *J. Neurosci. Res.* **83,** 1190-1200.

McMahon H. T., Missler M., Li C. and Sudhof T. C. (1995) Complexins - cytosolic proteins that regulate snap receptor function. *Cell* **83,** 111-119.

Mead C. and Pentreath V. W. (1998) Evaluation of toxicity indicators in rat primary astrocytes, C6 glioma and human 1321N1 astrocytoma cells: can gliotoxicity be distinguished from cytotoxicity? *Arch. Toxicol.* **72,** 372-380.

Mefford I. N. (1981) Application of high performance liquid chromatography with electrochemical detection to neurochemical analysis: measurement of catecholamines, serotonin and metabolites in rat brain. *J. Neurosci. Methods* **3,** 207-224.

Mehta M. A., Sahakian B. J., McKenna P. J. and Robbins T. W. (1999) Systemic sulpiride in young adult volunteers simulates the profile of cognitive deficits in Parkinson's disease. *Psychopharmacology (Berl).* **146,** 162-174.

Meister A., Morris H. P. and Tice S. V. (1953) Effect of vitamin B6 defiency on hepatic transaminase and cysteine desulfhydrase systems. *Proc. Soc. Exp. Biol. Med.* **82,** 301-304.

Meltzer H., Bastani B., Jayathilake K. and Maes M. (1997) Fluoxetine, but not tricyclic antidepressants, potentiates the 5-hydroxytryptophan-mediated increase in plasma cortisol and prolactin secretion in subjects with major depression or with obsessive compulsive disorder. *Neuropsychopharmacology* **17,** 1-11.

Mena M. A., Pardo B., Paino C. L. and De Yebenes J. G. (1993) Levodopa toxicity in foetal rat midbrain neurones in culture: modulation by ascorbic acid. *Neuroreport* **4,** 438-440.

Mena M. A., Casarejos M. J., Carazo A., Paino C. L. and Garcia d. Y. (1996) Glia conditioned medium protects fetal rat midbrain neurones in culture from L-DOPA toxicity. *Neuroreport* **7,** 441-445.

Mena M. A., Casarejos M. J., Carazo A., Paino C. L. and Garcia d. Y. (1997b) Glia protect fetal midbrain dopamine neurons in culture from L-DOPA toxicity through multiple mechanisms. *J. Neural Transm.* **104,** 317-328.

Mena M. A., Davila V. and Sulzer D. (1997a) Neurotrophic effects of L-DOPA in postnatal midbrain dopamine neuron/cortical astrocyte cocultures. *J. Neurochem.* **69,** 1398-1408.

Merad-Boudia M., Nicole A., Santiard-Baron D., Saille C. and Ceballos-Picot I. (1998) Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochem. Pharmacol.* **56,** 645-655.

Merad-Saidoune M., Boitier E., Nicole A., Marsac C., Martinou J. C., Sola B., Sinet P. M. and Ceballos-Picot I. (1999) Overproduction of Cu/Zn-superoxide dismutase or Bcl-2 prevents the brain mitochondrial respiratory dysfunction induced by glutathione depletion. *Exp. Neurol.* **158,** 428-436.

Miller J. E. and Litwack G. (1971) Purification, properties, and identity of liver mitochondrial tyrosine aminotransferase. *J. Biol. Chem.* **246,** 3234-3240.

Miller R. J. (1991) The control of neuronal Ca^{2+} homeostasis. *Prog. Neurobiol.* **37,** 255-285.

Mills K. C. (1995) Serotonin syndrome. *Am. Fam. Physician* **52,** 1475-1482.

Mills P. B., Surtees R. A. H., Champion M. P., Beesley C. E., Dalton N., Scambler P. J., Heales S. J. R., Briddon A., Scheimberg I., Hoffmann G. F., Zschocke J. and Clayton P. T. (2005) Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5 '-phosphate oxidase. *Hum. Mol. Genet.* **14,** 1077-1086.

Minc-Golomb D., Yadid G., Tsarfaty I., Resau J. H. and Schwartz J. P. (1996) In vivo expression of inducible nitric oxide synthase in cerebellar neurons. *J. Neurochem.* **66,** 1504-1509.

Minelli A., Conte C., Grottelli S., Bellezza M., Cacciatore I. and Bolanos J. P. (2009) Cyclo(His-Pro) promotes cytoprotection by activating Nrf2-mediated up-regulation of antioxidant defence. *J. Cell Mol. Med.* **13,** 1149-1161.

Mink J. W. and Thach W. T. (1993) Basal ganglia intrinsic circuits and their role in behavior. *Curr. Opin. Neurobiol.* **3,** 950-957.

Miodownik C., Meoded A., Libov I., Bersudsky Y., Sela B. A. and Lerner V. (2008) Pyridoxal plasma level in schizophrenic and schizoaffective patients with and without tardive dyskinesia. *Clin. Neuropharmacol.* **31,** 197-203.

Missale C., Nash S. R., Robinson S. W., Jaber M. and Caron M. G. (1998) Dopamine receptors: From structure to function. *Physiol. Rev.* **78,** 189-225.

Mitchell H. K. and Nyc J. F. (1948) Hydroxyantheranilic acid as a precursor of nicotinic acid in Neurospora. *Proc. Natl. Acad. Sci. U. S. A.* **34,** 1-5.

Mitoma C. (1956) Studies on partially purified phenylalanine hydroxylase. *Arch. Biochem. Biophys.* **60,** 476-484.

Mokler D. J., Sullivan S. A. and Winterson B. J. (1992) Behaviors induced by 5-hydroxytryptophan in neonatal, preweaning, postweaning, and adult Sprague-Dawley rats. *Pharmacol. Biochem. Behav.* **42,** 413-419.

Molnar I. and Horvath C. (1976) Reverse-phase chromatography of polar biological substances: separation of catechol compounds by high-performance liquid chromatography. *Clin. Chem.* **22,** 1497-1502.

Molodtsova G. F. (2008) Serotonergic mechanisms of memory trace retrieval. *Behav. Brain Res.* **195,** 7-16.

Monder C., Williams J. N. and Waisman H. A. (1957a) Studies on the non-enzymic conversion of dopa to melanin .1. studies on autoxidation. *Arch. Biochem. Biophys.* **72,** 255-270.

Monder C., Williams J. N. and Waisman H. A. (1957b) The non-enzymic conversion of dopa to melanin .2. the aerobic catalysis of dopa oxidation by copper ions and copper-plasma protein complexes. *Arch. Biochem. Biophys.* **72,** 271-292.

Monti J. M. (1968) The effect of haloperidol on the sleep cycle of the cat. *Experientia* **24,** 1143.

Moore D. J., Zhang L., Dawson T. M. and Dawson V. L. (2003) A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. *J. Neurochem.* **87,** 1558-1567.

Moore P. S., Dominici P. and Voltattorni C. B. (1996) Cloning and expression of pig kidney dopa decarboxylase: Comparison of the naturally occurring and recombinant enzymes. *Biochem. J.* **315,** 249-256.

Morita T., Saito K., Takemura M., Maekawa N., Fujigaki S., Fujii H., Wada H., Takeuchi S., Noma A. and Seishima M. (2001) 3-Hydroxyanthranilic acid, an L-tryptophan metabolite, induces apoptosis in monocyte-derived cells stimulated by interferon-gamma. *Ann. Clin. Biochem.* **38,** 242-251.

Moriyama Y. and Nelson N. (1987) The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. *J. Biol. Chem.* **262,** 9175-9180.

Moroni F., Cozzi A., Peruginelli F., Carpenedo R. and Pellegrini-Giampietro D. E. (1999) Neuroprotective effects of kynurenine-3-hydroxylase inhibitors in models of brain ischemia. *Adv. Exp. Med. Biol.* **467,** 199-206.

Motohashi H. and Yamamoto M. (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* **10,** 549-557.

Mrak R. E. and Griffin W. S. (2005) Glia and their cytokines in progression of neurodegeneration. *Neurobiol. Aging* **26,** 349-354.

Mulcahy R. T. and Gipp J. J. (1995) Identification of a putative antioxidant response element in the 5'-flanking region of the human gamma-glutamylcysteine synthetase heavy subunit gene. *Biochem. Biophys. Res. Commun.* **209,** 227-233.

Muller T., Hefter H., Hueber R., Jost W. H., Leenders K. L., Odin P. and Schwarz J. (2004) Is levodopa toxic? *J. Neurol.* **251,** 44-46.

Munemura M., Eskay R. L. and Kebabian J. W. (1980) Release of alpha-melanocyte-stimulating hormone from dispersed cells of the intermediate lobe of the rat pituitary-gland - involvement of catecholamines and adenosine-3',5'-monophosphate. *Endocrinology* **106,** 1795-1803.

Munn D. H., Shafizadeh E., Attwood J. T., Bondarev I., Pashine A. and Mellor A. L. (1999) Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* **189,** 1363-1372.

Munoz-Castaneda J. R., Montilla P., Munoz M. C., Bujalance I., Muntane J. and Tunez I. (2005) Parachlorophenylalanine exacerbates oxidative stress induced by gentamicin in rats. *Basic Clin. Pharmacol. Toxicol.* **97,** 174-178.

Munoz-Castaneda J. R., Montilla P., Padillo F. J., Bujalance I., Munoz M. C., Muntane J. and Tunez I. (2006) Role of serotonin in cerebral oxidative stress in rats. *Acta Neurobiol. Exp. (Wars.)* **66,** 1-6.

Munoz-Fernandez M. A., Cano E., O'Donnell C. A., Doyle J., Liew F. Y. and Fresno M. (1994) Tumor necrosis factor-alpha (TNF-alpha), interferon-gamma, and interleukin-6

but not TNF-beta induce differentiation of neuroblastoma cells: the role of nitric oxide. *J. Neurochem.* **62,** 1330-1336.

Murer M. G., Dziewczapolski G., Menalled L. B., Garcia M. C., Agid Y., Gershanik O. and Raisman-Vozari R. (1998) Chronic levodopa is not toxic for remaining dopamine neurons, but instead promotes their recovery, in rats with moderate nigrostriatal lesions. *Ann. Neurol.* **43,** 561-575.

Mytilineou C., Han S. K. and Cohen G. (1993) Toxic and protective effects of L-dopa on mesencephalic cell cultures. *J. Neurochem.* **61,** 1470-1478.

Mytilineou C., Walker R. H., JnoBaptiste R. and Olanow C. W. (2003) Levodopa is toxic to dopamine neurons in an in vitro but not an in vivo model of oxidative stress. *J. Pharmacol. Exp. Ther.* **304,** 792-800.

Nagatsu T., Levitt M. and Udenfriend S. (1964) Tyrosine hydroxylase - initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239,** 2910-2917.

Nagatsu T., Yamamoto T. and Kato T. (1979) A new and highly sensitive voltammetric assay for aromatic L-amino acid decarboxylase activity by high-performance liquid chromatography. *Anal. Biochem.* **100,** 160-165.

Naito J., Sasaki E., Ohta Y., Shinohara R. and Ishiguro I. (1984) Anthranilic acid metabolism in the isolated perfused rat liver: detection and determination of anthranilic acid and its related substances using high-performance liquid chromatography with electrochemical detection. *Biochem. Pharmacol.* **33,** 3195-3200.

Nakamura M., Ishii A. and Nakahara D. (1998) Characterization of beta-phenylethylamine-induced monoamine release in rat nucleus accumbens: a microdialysis study. *Eur. J. Pharmacol.* **349,** 163-169.

Nakao N., Nakai K. and Itakura T. (1997) Metabolic inhibition enhances selective toxicity of L-DOPA toward mesencephalic dopamine neurons in vitro. *Brain Res.* **777,** 202-209.

Naoi M., Maruyama W., Yi H., Akao Y., Yamaoka Y. and Shamoto-Nagai M. (2007) Neuroprotection by propargylamines in Parkinson's disease: intracellular mechanism underlying the anti-apoptotic function and search for clinical markers. *J. Neural Transm. Suppl.* 121-131.

Nardini M., De Stefano R., Iannuccelli M., Borghesi R. and Battistini N. (1983) Treatment of depression with L-5-hydroxytryptophan combined with chlorimipramine, a double-blind study. *Int. J. Clin. Pharmacol. Res.* **3,** 239-250.

Nasrin S., Ichinose H. and Nagatsu T. (1992) Comparison of characteristics of bovine aromatic L-amino-acid decarboxylase with human enzyme. *Biochim. Biophys. Acta* **1118,** 318-322.

Neame S. J., Rubin L. L. and Philpott K. L. (1998) Blocking cytochrome c activity within intact neurons inhibits apoptosis. *J. Cell Biol.* **142,** 1583-1593.

Neckers L. M., Biggio G., Moja E. and Meek J. L. (1977) Modulation of brain tryptophan hydroxylase activity by brain tryptophan content. *J. Pharmacol. Exp. Ther.* **201,** 110-116.

Neff N. H., Wemlinger T. A., Duchemin A. M. and Hadjiconstantinou M. (2006) Clozapine modulates aromatic L-amino acid decarboxylase activity in mouse striatum. *J. Pharmacol. Exp. Ther.* **317,** 480-487.

Ng T. B., Liu F. and Zhao L. (2000) Antioxidative and free radical scavenging activities of pineal indoles. *J. Neural Transm.* **107,** 1243-1251.

Nicholls D. G. and Budd S. L. (2000) Mitochondria and neuronal survival. *Physiol Rev.* **80,** 315-360.

Nicole A., Santiard-Baron D. and Ceballos-Picot I. (1998) Direct evidence for glutathione as mediator of apoptosis in neuronal cells. *Biomed. Pharmacother.* **52,** 349-355.

Niederwieser A., Blau N., Wang M., Joller P., Atares M. and Cardesagarcia J. (1984) GTP cyclohydrolase-I deficiency, A new enzyme defect causing hyperphenylalaninemia with neopterin, biopterin, dopamine, and serotonin deficiencies and muscular hypotonia. *Eur. J. Pediatr.* **141,** 208-214.

Niederwieser A., Leimbacher W., Curtius H. C., Ponzone A., Rey F. and Leupold D. (1985) Atypical phenylketonuria with "dihydrobiopterin synthetase deficiency" - absence of phosphate-eliminating enzyme-activity demonstrated in liver. *Eur. J. Pediatr.* **144,** 13-16.

Nirenberg M. J., Vaughan R. A., Uhl G. R., Kuhar M. J. and Pickel V. M. (1996) The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J. Neurosci.* **16,** 436-447.

Nishizuka Y. and Hayaishi O. (1963) Enzymic synthesis of niacin nucleotides from 3-hydroxyanthranilic acid in mammalian liver. *J. Biol. Chem.* **238,** 483-485.

Nisijima K., Yoshino T., Yui K. and Katoh S. (2001) Potent serotonin (5-HT)(2A) receptor antagonists completely prevent the development of hyperthermia in an animal model of the 5-HT syndrome. *Brain Res.* **890,** 23-31.

Noguchi T., Kido R. and Matsumura Y. (1969) Enzymic formation of 6-hydroxykynurenic acid and 5-hydroxyanthranilic acid from 5-hydroxykynurenine. *Wakayama. Med. Rep.* **13,** 149-154.

Noguchi T., Kaseda H., Kido R. and Matsumura Y. (1970) 5-hydroxykynurenine decarboxylase in rat intestine. *J. Biochem.* **67,** 113-121.

Nonogaki K. (2000) New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* **43,** 533-549.

O'Malley K. L., Harmon S., Moffat M., Uhland-Smith A. and Wong S. (1995) The human aromatic L-amino acid decarboxylase gene can be alternatively spliced to generate unique protein isoforms. *J. Neurochem.* **65,** 2409-2416.

Offen D., Panet H., Galili-Mosberg R. and Melamed E. (2001) Catechol-O-methyltransferase decreases levodopa toxicity in vitro. *Clin. Neuropharmacol.* **24,** 27-30.

Okado-Matsumoto A. and Fridovich I. (2001) Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J. Biol. Chem.* **276,** 38388-38393.

Okuda S., Nishiyama N., Saito H. and Katsuki H. (1998) 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. *J. Neurochem.* **70,** 299-307.

Okuma M., Tokuyama T., Senoh S., Hirata F. and Hayaishi O. (1976) Antagonism of 5-hydroxykynurenamine against serotonin action on platelet aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **73,** 643-645.

Oleary M. H. and Baughn R. L. (1977) Decarboxylation-dependent transamination catalyzed by mammalian 3,4-dihydroxyphenylalanine decarboxylase. *J. Biol. Chem.* **252,** 7168-7173.

Omar R. and Pappolla M. (1993) Oxygen free radicals as inducers of heat shock protein synthesis in cultured human neuroblastoma cells: relevance to neurodegenerative disease. *Eur. Arch. Psychiatry Clin. Neurosci.* **242,** 262-267.

Onali P., Olianas M. C. and Gessa G. L. (1984) Selective blockade of dopamine D-1 receptors by SCH 23390 discloses striatal dopamine D-2 receptors mediating the inhibition of adenylate cyclase in rats. *Eur. J. Pharmacol.* **99,** 127-128.

Oppenheim M. L., Hargreaves I. P., Pope S., Land J. M. and Heales S. J. (2009) Mitochondrial cytochrome c release: a factor to consider in mitochondrial disease? *J. Inherit. Metab. Dis.* **32,** 269-273.

Oyarce A. M. and Fleming P. J. (1991) Multiple forms of human dopamine beta-hydroxylase in SH-SY5Y neuroblastoma cells. *Arch. Biochem. Biophys.* **290,** 503-510.

Packer M. A., Miesel R. and Murphy M. P. (1996) Exposure to the parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP+) and nitric oxide simultaneously causes cyclosporin A-sensitive mitochondrial calcium efflux and depolarisation. *Biochem. Pharmacol.* **51,** 267-273.

Pardo B., Mena M. A., Casarejos M. J., Paino C. L. and De Yebenes J. G. (1995) Toxic effects of L-DOPA on mesencephalic cell cultures: protection with antioxidants. *Brain Res.* **682,** 133-143.

Park I. H., Lerou P. H., Zhao R., Huo H. and Daley G. Q. (2008a) Generation of human-induced pluripotent stem cells. *Nat. Protoc.* **3,** 1180-1186.

Park I. H., Zhao R., West J. A., Yabuuchi A., Huo H., Ince T. A., Lerou P. H., Lensch M. W. and Daley G. Q. (2008b) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451,** 141-146.

Pattison D. I., Dean R. T. and Davies M. J. (2002) Oxidation of DNA, proteins and lipids by DOPA, protein-bound DOPA, and related catechol(amine)s. *Toxicology* **177,** 23-37.

Pearl P. L., Taylor J. L., Trzcinski S. and Sokohl A. (2007) The pediatric neurotransmitter disorders. *J. Child Neurol.* **22,** 606-616.

Pedrosa R. and Soares-Da-Silva P. (2002) Generation of L-DOPA quinones and H_2O_2 as major mechanisms in L-DOPA induced neuronal death: Implications in Parkinson's disease. *Br. J. Pharmacol.* **135**.

Pena C., Zhou Y., Lust D. and Pilar G. (2001) Restoration of mitochondrial function reverses developmental neuronal death *in vitro*. *J. Comp Neurol.* **440,** 156-176.

Penrose L. S. (1935) Inheritance of phenylpyruvic amentia (phenylketonuria). *Lancet* **226,** 192-194.

Perry T. L., Yong V. W., Ito M., Foulks J. G., Wall R. A., Godin D. V. and Clavier R. M. (1984) Nigrostriatal dopaminergic neurons remain undamaged in rats given high doses of L-DOPA and carbidopa chronically. *J. Neurochem.* **43,** 990-993.

Pestana M., Jardim H., Correia F., Vieira-Coelho M. A. and Soares-Da-Silva P. (2001) Renal dopaminergic mechanisms in renal parenchymal diseases and hypertension. *Nephrol. Dial. Transplant.* **16 Suppl 1,** 53-59.

Peter D., Liu Y., Sternini C., de Giorgio R., Brecha N. and Edwards R. H. (1995) Differential expression of two vesicular monoamine transporters. *J. Neurosci.* **15,** 6179-6188.

Petit J. F., Schott C., Lepoivre M. and Stoclet J. C. (1990) Production of an arginine-derived relaxing factor induced by IFN-gamma plus endotoxin in murine adenocarcinoma EMT 6 cells. *Biochem. Biophys. Res. Commun.* **173,** 1-5.

Peuchen S., Bolanos J. P., Heales S. J., Almeida A., Duchen M. R. and Clark J. B. (1997) Interrelationships between astrocyte function, oxidative stress and antioxidant status within the central nervous system. *Prog. Neurobiol.* **52,** 261-281.

Picetti R., Saiardi A., Abdel S. T., Bozzi Y., Baik J. H. and Borrelli E. (1997) Dopamine D2 receptors in signal transduction and behavior. *Crit Rev. Neurobiol.* **11,** 121-142.

Pletscher A., Bartholini G. and Tissot R. (1967) Metabolic fate of l-[14C] DOPA in cerebrospinal fluid and blood plasma of humans. *Brain Res.* **4,** 106-109.

Poeggeler B., Thuermann S., Dose A., Schoenke M., Burkhardt S. and Hardeland R. (2002) Melatonin's unique radical scavenging properties - roles of its functional substituents as revealed by a comparison with its structural analogs. *J. Pineal Res.* **33,** 20-30.

Pompeiano M., Palacios J. M. and Mengod G. (1994) Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A) and 5-HT(2C) receptors. *Brain Res. Mol. Brain Res.* **23,** 163-178.

Pons R., Ford B., Chiriboga C. A., Clayton P. T., Hinton V., Hyland K., Sharma R. and De Vivo D. C. (2004) Aromatic L-amino acid decarboxylase deficiency - Clinical features, treatment, and prognosis. *Neurology* **62,** 1058-1065.

Ponten J. and Macintyre E. H. (1968) Long term culture of normal and neoplastic human glia. *Acta Pathol. Microbiol. Scand.* **74,** 465-486.

Pope S. A., Milton R. and Heales S. J. (2008) Astrocytes protect against copper-catalysed loss of extracellular glutathione. *Neurochem. Res.* **33,** 1410-1418.

Proudfit H. K. and Hammond D. L. (1981) Alterations in nociceptive threshold and morphine-induced analgesia produced by intrathecally administered amine antagonists. *Brain Res.* **218,** 393-399.

Quinn N., Parkes D., Janota I. and Marsden C. D. (1986) Preservation of the substantia nigra and locus coeruleus in a patient receiving levodopa (2 kg) plus decarboxylase inhibitor over a four-year period. *Mov. Disord.* **1,** 65-68.

Rafice S. A., Chauhan N., Efimov I., Basran J. and Raven E. L. (2009) Oxidation of L-tryptophan in biology: a comparison between tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase. *Biochem. Soc. Trans.* **37,** 408-412.

Ragan C. I., Wilson M. T., Darley-Usmar V. M. and Lowe P. N. (1987) Subfractionation of mitochondria and isolation of proteins of oxidative phosphorylation, in *Mitochondria. A practical approach,* (Darley-Usmar V. M., Rickwood D. and Wilson M. T., eds), pp. 79-112. IRL, Oxford.

Rahman I., Bel A., Mulier B., Lawson M. F., Harrison D. J., Macnee W. and Smith C. A. (1996) Transcriptional regulation of gamma-glutamylcysteine synthetase-heavy

subunit by oxidants in human alveolar epithelial cells. *Biochem. Biophys. Res. Commun.* **229,** 832-837.

Rahman M. K., Nagatsu T., Sakurai T., Hori S., Abe M. and Matsuda M. (1982) Effect of pyridoxal phosphate deficiency on aromatic L-amino acid decarboxylase activity with L-dopa and L-5-hydroxytryptophan as substrates in rats. *Jpn. J. Pharmacol.* **32,** 803-811.

Rall T. W. and Lehninger A. L. (1952) Glutathione reductase of animal tissues. *J. Biol. Chem.* **194,** 119-130.

Ramaekers V. T., Senderek J., Hausler M., Haring M., Abeling N., Zerres K., Bergmann C., Heimann G. and Blau N. (2001) A novel neurodevelopmental syndrome responsive to 5-hydroxytryptophan and carbidopa. *Mol. Genet. Metab.* **73,** 179-187.

Rapport M. M., Green A. A. and Page I. H. (1948) Serum vasoconstrictor (serotonin) .4. Isolation and characterization. *J. Biol. Chem.* **176,** 1243-1251.

Raps S. P., Lai J. C., Hertz L. and Cooper A. J. (1989) Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. *Brain Res.* **493,** 398-401.

Reches A. and Fahn S. (1982) 3-O-methyldopa blocks dopa metabolism in rat corpus striatum. *Ann. Neurol.* **12,** 267-271.

Reim K., Mansour M., Varoqueaux F., McMahon H. T., Sudhof T. C., Brose N. and Rosenmund C. (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. *Cell* **104,** 71-81.

Ridge J., Terle D. A., Dragunsky E. and Levenbook I. (1996) Effects of gamma-IFN and NGF on subpopulations in a human neuroblastoma cell line: flow cytometric and morphological analysis. *In Vitro Cell. Dev. Biol. Anim.* **32,** 238-248.

Riedel W. J., Klaassen T., Deutz N. E. P., van Someren A. and van Praag H. M. (1999) Tryptophan depletion in normal volunteers produces selective impairment in memory consolidation. *Psychopharmacology (Berl).* **141,** 362-369.

Riederer P., Sofic E., Rausch W. D., Schmidt B., Reynolds G. P., Jellinger K. and Youdim M. B. (1989) Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. *J. Neurochem.* **52,** 515-520.

Riley P. A. (1997) Melanin. *Int. J. Biochem. Cell Biol.* **29,** 1235-1239.

Robbins T. W. and Everett B. J. (2002) Dopamine- its role in behaviour and cognition in experimental animals and humans, in *Dopamine in the CNS II,* (Di Chiara G., ed),Vol. 154, pp. 173-211. Springer-Verlag, Berlin.

Robertson D., Haile V., Perry S. E., Robertson R. M., Phillips J. A. and Biaggioni I. (1991) Dopamine beta-hydroxylase deficiency. A genetic disorder of cardiovascular regulation. *Hypertension* **18,** 1-8.

Rosengren E. (1960) On the role of monoamine oxidase for the inactivation of dopamine in brain. *Acta Physiol. Scand.* **49,** 370-375.

Ross R. A., Joh T. H., Reis D. J., Spengler B. A. and Biedler J. L. (1980) Neurotransmitter-synthesizing enzymes in human neuroblastoma cells: relationship to morphological diversity, in *Advances in neuroblastoma research,* (Evans A. E., ed), pp. 151-160. Raven Press, New York.

Rossetti Z., Krajnc D., Neff N. H. and Hadjiconstantinou M. (1989) Modulation of retinal aromatic L-amino acid decarboxylase via alpha-2-adrenoceptors. *J. Neurochem.* **52,** 647-652.

Rossetti Z. L., Silvia C. P., Krajnc D., Neff N. H. and Hadjiconstantinou M. (1990) Aromatic L-amino acid decarboxylase is modulated by D1 dopamine receptors in rat retina. *J. Neurochem.* **54,** 787-791.

Roy E. J., Takikawa O., Kranz D. M., Brown A. R. and Thomas D. L. (2005) Neuronal localization of indoleamine 2,3-dioxygenase in mice. *Neurosci. Lett.* **387,** 95-99.

Rushmore T. H., Morton M. R. and Pickett C. B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266,** 11632-11639.

Russo V. C., Kobayashi K., Najdovska S., Baker N. L. and Werther G. A. (2004) Neuronal protection from glucose deprivation via modulation of glucose transport and inhibition of apoptosis: a role for the insulin-like growth factor system. *Brain Res.* **1009,** 40-53.

Ruth J. A., Cuizon J. V., Park S. H., Ullman E. A. and Wilson W. R. (1986) 3H-5-hydroxytryptamine accumulation by rat brain synaptic vesicles in a membrane-impermeant medium, and selective reduction by 5,7-dihydroxytryptamine. *Life Sci.* **38,** 1193-1201.

Sagara J., Makino N. and Bannai S. (1996) Glutathione efflux from cultured astrocytes. *J. Neurochem.* **66,** 1876-1881.

Sagara J. I., Miura K. and Bannai S. (1993) Maintenance of neuronal glutathione by glial cells. *J. Neurochem.* **61,** 1672-1676.

Saller C. F. and Stricker E. M. (1976) Hyperphagia and increased growth in rats after intraventricular-injection of 5,7-dihydroxytryptamine. *Science* **192,** 385-387.

Samikkannu T., Rao K. V., Gandhi N., Saxena S. K. and Nair M. P. (2010) Human immunodeficiency virus type 1 clade B and C Tat differentially induce indoleamine 2,3-dioxygenase and serotonin in immature dendritic cells: Implications for neuroAIDS. *J. Neurovirol.* **16,** 256-264.

Sandyk R. and Pardeshi R. (1990) Pyridoxine improves drug-induced parkinsonism and psychosis in a schizophrenic patient. *Int. J. Neurosci.* **52,** 225-232.

Santamaria A., Jimenez-Capdeville M. E., Camacho A., Rodriguez-Martinez E., Flores A. and Galvan-Arzate S. (2001) In vivo hydroxyl radical formation after quinolinic acid infusion into rat corpus striatum. *Neuroreport* **12,** 2693-2696.

Sas K., Robotka H., Toldi J. and Vecsei L. (2007) Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *J. Neurol. Sci.* **257,** 221-239.

Sathyasaikumar K. V., Stachowski E. K., Amori L., Guidetti P., Muchowski P. J. and Schwarcz R. (2010) Dysfunctional kynurenine pathway metabolism in the R6/2 mouse model of Huntington's disease. *J. Neurochem.* **113,** 1416-1425.

Sawicki J., Kuzma M. and Baranczyk-Kuzma A. (2001) The effect of serotonin, its precursors and metabolites on brain glutathione-S-transferase. *Neurochem. Res.* **26,** 469-472.

Scatena R., Martorana G. E., Bottoni P., Botta G., Pastore P. and Giardina B. (2007) An update on pharmacological approaches to neurodegenerative diseases. *Expert. Opin. Investig. Drugs* **16,** 59-72.

Schapira A. H., Emre M., Jenner P. and Poewe W. (2009) Levodopa in the treatment of Parkinson's disease. *Eur. J. Neurol.* **16,** 982-989.

Schmidt C. J., Abbate G. M., Black C. K. and Taylor V. L. (1990) Selective 5-hydroxytryptamine2 receptor antagonists protect against the neurotoxicity of methylenedioxymethamphetamine in rats. *J. Pharmacol. Exp. Ther.* **255,** 478-483.

Schmuck K., Ullmer C., Engels P. and Lubbert H. (1994) Cloning and functional characterization of the human 5-HT2B serotonin receptor. *FEBS Lett.* **342,** 85-90.

Schultz J. R., Ellerby L. M., Gralla E. B., Valentine J. S. and Clarke C. F. (1996) Autoxidation of ubiquinol-6 is independent of superoxide dismutase. *Biochemistry (Mosc).* **35,** 6595-6603.

Schurr A., West C. A. and Rigor B. M. (1991) Neurotoxicity of quinolinic acid and its derivatives in hypoxic rat hippocampal slices. *Brain Res.* **568,** 199-204.

Schwarcz R., Guidetti P., Sathyasaikumar K. V. and Muchowski P. J. (2010) Of mice, rats and men: Revisiting the quinolinic acid hypothesis of Huntington's disease. *Prog. Neurobiol.* **90,** 230-245.

Scott E. M., Duncan I. W. and Ekstrand V. (1963) Purification and properties of glutathione reductase of human erythrocytes. *J. Biol. Chem.* **238,** 3928-3933.

Seamans J. K. and Yang C. R. (2004) The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Prog. Neurobiol.* **74,** 1-57.

Segura-Aguilar J. and Lind C. (1989) On the mechanism of the Mn3(+)-induced neurotoxicity of dopamine:prevention of quinone-derived oxygen toxicity by DT diaphorase and superoxide dismutase. *Chem. Biol. Interact.* **72,** 309-324.

Seiji M. and Fitzpatrick T. B. (1961) The reciprocal relationship between melanization and tyrosinase activity in melanosomes (melanin granules). *J. Biochem.* **49,** 700-706.

Seiji M., Fitzpatrick T. B., Simpson R. T. and Birbeck M. S. (1963) Chemical composition and terminology of specialized organelles (melanosomes and melanin granules) in mammalian melanocytes. *Nature* **197,** 1082-1084.

Sheard M. H. (1969) The effect of p-chlorophenylalanine on behavior in rats: relation to brain serotonin and 5-hydroxyindoleacetic acid. *Brain Res.* **15,** 524-528.

Shepherd D. and Garland P. B. (1969) The kinetic properties of citrate synthase from rat liver mitochondria. *Biochem. J.* **114,** 597-610.

Sherald A. F., Sparrow J. C. and Wright T. R. (1973) A spectrophotometric assay for Drosophila dopa decarboxylase. *Anal. Biochem.* **56,** 300-305.

Shih A. Y., Erb H. and Murphy T. H. (2007) Dopamine activates Nrf2-regulated neuroprotective pathways in astrocytes and meningeal cells. *J. Neurochem.* **101,** 109-119.

Shimada S., Kitayama S., Lin C. L., Patel A., Nanthakumar E., Gregor P., Kuhar M. and Uhl G. (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary-DNA. *Science* **254,** 576-578.

Shimizu T., Nomiyama S., Hirata F. and Hayaishi O. (1978) Indoleamine 2,3-dioxygenase. Purification and some properties. *J. Biol. Chem.* **253,** 4700-4706.

Shintaku H. (2002) Disorders of tetrahydrobiopterin metabolism and their treatment. *Curr. Drug Metab.* **3,** 123-131.

Simansky K. J. and Vaidya A. H. (1990) Behavioral mechanisms for the anorectic action of the serotonin (5-HT) uptake inhibitor sertraline in rats - comparison with directly acting 5-HT agonists. *Brain Res. Bull.* **25,** 953-960.

Simansky K. J. (1996) Serotonergic control of the organization of feeding and satiety. *Behav. Brain Res.* **73,** 37-42.

Siow Y. L. and Dakshinamurti K. (1985) Effect of pyridoxine deficiency on aromatic L-amino acid decarboxylase in adult rat brain. *Exp. Brain Res.* **59,** 575-581.

Sjoerdsma A., Smith T. F., Stevenson T. D. and Udenfriend S. (1955) Metabolism of 5-hydroxytryptamine (serotonin) by monoamine oxidase. *Proc. Soc. Exp. Biol. Med.* **89,** 36-38.

Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney S. and Boyd M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82,** 1107-1112.

Skou J. C. (1998) Nobel Lecture. The identification of the sodium pump. *Biosci. Rep.* **18,** 155-169.

Slivka A., Mytilineou C. and Cohen G. (1987) Histochemical evaluation of glutathione in brain. *Brain Res.* **409,** 275-284.

Smith A. J., Smith R. A. and Stone T. W. (2009) 5-Hydroxyanthranilic acid, a tryptophan metabolite, generates oxidative stress and neuronal death via p38 activation in cultured cerebellar granule neurones. *Neurotox. Res.* **15,** 303-310.

Smith T. S., Parker W. D., Jr. and Bennett J. P., Jr. (1994) L-dopa increases nigral production of hydroxyl radicals in vivo: potential L-dopa toxicity? *Neuroreport* **5,** 1009-1011.

Snape B. M., Badawy A. A. B. and Evans M. (1980) Stabilization of rat liver tyrosine aminotransferase *in vivo* by pyridoxine administration. *Biochem. J.* **186,** 625-627.

Snoke J. E. and Bloch K. (1955) Studies on the mechanism of action of glutathione synthetase. *J. Biol. Chem.* **213,** 825-835.

Soares-Da-Silva P. and Fernandes M. H. (1990) Regulation of dopamine synthesis in the rat kidney. *J. Auton. Pharmacol.* **10 Suppl 1,** s25-s30.

Sollner T., Whitehart S. W., Brunner M., Erdjumentbromage H., Geromanos S., Tempst P. and Rothman J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362,** 318-324.

Spencer J. P., Jenner A., Butler J., Aruoma O. I., Dexter D. T., Jenner P. and Halliwell B. (1996) Evaluation of the pro-oxidant and antioxidant actions of L-DOPA and dopamine in vitro: implications for Parkinson's disease. *Free Radic. Res.* **24,** 95-105.

Spina M. B. and Cohen G. (1989) Dopamine turnover and glutathione oxidation: implications for Parkinson disease. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1398-1400.

Steenken S. (1989) Purine bases, nucleosides, and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e- and OH adducts. *Chem. Rev.* **89,** 503-520.

Stewart C. W., Bowyer J. F. and Slikker W. (1997) Elevated environmental temperatures can induce hyperthermia during d-fenfluramine exposure and enhance 5-

hydroxytryptamine (5-HT) depletion in the brain. *J. Pharmacol. Exp. Ther.* **283,** 1144-1150.

Stewart V. C., Land J. M., Clark J. B. and Heales S. J. (1998) Pretreatment of astrocytes with interferon-alpha/beta prevents neuronal mitochondrial respiratory chain damage. *J. Neurochem.* **70,** 432-434.

Stewart V. C., Sharpe M. A., Clark J. B. and Heales S. J. (2000) Astrocyte-derived nitric oxide causes both reversible and irreversible damage to the neuronal mitochondrial respiratory chain. *J. Neurochem.* **75,** 694-700.

Stewart V. C., Stone R., Gegg M. E., Sharpe M. A., Hurst R. D., Clark J. B. and Heales S. J. (2002) Preservation of extracellular glutathione by an astrocyte derived factor with properties comparable to extracellular superoxide dismutase. *J. Neurochem.* **83,** 984-991.

Stock D., Leslie A. G. and Walker J. E. (1999) Molecular architecture of the rotary motor in ATP synthase. *Science* **286,** 1700-1705.

Stock D., Gibbons C., Arechaga I., Leslie A. G. and Walker J. E. (2000) The rotary mechanism of ATP synthase. *Curr. Opin. Struct. Biol.* **10,** 672-679.

Stone J. M., Morrison P. D. and Pilowsky L. S. (2007a) Glutamate and dopamine dysregulation in schizophrenia - A synthesis and selective review. *J. Psychopharmacol. (Oxf).* **21,** 440-452.

Stone R., Stewart V. C., Hurst R. D., Clark J. B. and Heales S. J. (1999) Astrocyte nitric oxide causes neuronal mitochondrial damage, but antioxidant release limits neuronal cell death. *Ann. N. Y. Acad. Sci.* **893,** 400-403.

Stone T. W. and Perkins M. N. (1981) Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS. *Eur. J. Pharmacol.* **72,** 411-412.

Stone T. W., Forrest C. M., Mackay G. M., Stoy N. and Darlington L. G. (2007b) Tryptophan, adenosine, neurodegeneration and neuroprotection. *Metab. Brain Dis.* **22,** 337-352.

Storch A., Blessing H., Bareiss M., Jankowski S., Ling Z. D., Carvey P. and Schwarz J. (2000) Catechol-O-methyltransferase inhibition attenuates levodopa toxicity in mesencephalic dopamine neurons. *Mol. Pharmacol.* **57,** 589-594.

Sudhof T. C. (2004) The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27,** 509-547.

Sulzer D., Bogulavsky J., Larsen K. E., Behr G., Karatekin E., Kleinman M. H., Turro N., Krantz D., Edwards R. H., Greene L. A. and Zecca L. (2000) Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 11869-11874.

Sumi-Ichinose C., Ichinose H., Takahashi E., Hori T. and Nagatsu T. (1992) Molecular cloning of genomic DNA and chromosomal assignment of the gene for human aromatic L-amino acid decarboxylase, the enzyme for catecholamine and serotonin biosynthesis. *Biochemistry (Mosc).* **31,** 2229-2238.

Surtees R. and Hyland K. (1990) L-3,4-dihydroxyphenylalanine (levodopa) lowers central nervous system S-adenosylmethionine concentrations in humans. *J. Neurol. Neurosurg. Psychiatry* **53,** 569-572.

Surtees R. and Blau N. (2000) The neurochemistry of phenylketonuria. *Eur. J. Pediatr.* **159 Suppl 2,** S109-S113.

Suzuki O. and Yagi K. (1976) Fluorometric assay for beta-phenylethylamine in rat brain. *Anal. Biochem.* **75,** 192-200.

Swoboda K. J., Hyland K., Goldstein D. S., Kuban K. C. K., Arnold L. A., Holmes C. S. and Levy H. L. (1999) Clinical and therapeutic observations in aromatic L-amino acid decarboxylase deficiency. *Neurology* **53,** 1205-1211.

Swoboda K. J., Saul J. P., McKenna C. E., Speller N. B. and Hyland K. (2003) Aromatic L-amino acid decarboxylase deficiency - Overview of clinical features and outcomes. *Ann. Neurol.* **54,** S49-S55.

Taber E., Brodal A. and Walberg F. (1960) The raphe nuclei of the brain stem in the cat .1. Normal topography and cytoarchitecture and general discussion. *J. Comp. Neurol.* **114,** 161-187.

Takada M., Ikenoya S., Yuzuriha T. and Katayama K. (1984) Simultaneous determination of reduced and oxidized ubiquinones. *Methods Enzymol.* **105,** 147-155.

Takahashi K., Tanabe K., Ohnuki M., Narita M., Ichisaka T., Tomoda K. and Yamanaka S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861-872.

Takikawa O., Kuroiwa T., Yamazaki F. and Kido R. (1988) Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *J. Biol. Chem.* **263,** 2041-2048.

Tallman J. F., Saavedra J. M. and Axelrod J. (1976) Sensitive enzymatic isotopic method for analysis of tyramine in brain and other tissues. *J. Neurochem.* **27,** 465-469.

Tan D., Reiter R. J., Chen L. D., Poeggeler B., Manchester L. C. and Barlow-Walden L. R. (1994) Both physiological and pharmacological levels of melatonin reduce DNA adduct formation induced by the carcinogen safrole. *Carcinogenesis* **15,** 215-218.

Tan E. K., Cheah S. Y., Fook-Chong S., Yew K., Chandran V. R., Lum S. Y. and Yi Z. (2005) Functional COMT variant predicts response to high dose pyridoxine in Parkinson's disease. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **137B,** 1-4.

Tay S. K. H., Poh K. S., Hyland K., Pang Y. W., Ong H. T., Low P. S. and Goh D. L. M. (2007) Unusually mild phenotype of AADC deficiency in 2 siblings. *Mol. Genet. Metab.* **91,** 374-378.

Tecott L. H., Maricq A. V. and Julius D. (1993) Nervous system distribution of the serotonin 5-HT_3 receptor mRNA. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1430-1434.

Thomas S. R., Mohr D. and Stocker R. (1994) Nitric oxide inhibits indoleamine 2,3-dioxygenase activity in interferon-gamma primed mononuclear phagocytes. *J. Biol. Chem.* **269,** 14457-14464.

Tison F., Mons N., Geffard M. and Henry P. (1991) The metabolism of exogenous L-dopa in the brain: an immunohistochemical study of its conversion to dopamine in non-catecholaminergic cells of the rat brain. *J. Neural Transm. Park Dis. Dement. Sect.* **3,** 27-39.

Tissari A. H., Schonhof P. S., Bogdansk D. F. and Brodie B. B. (1969) Mechanism of biogenic amine transport .2. Relationship between sodium and mechanism of ouabain blockade of accumulation of serotonin and norepinephrine by synaptosomes. *Mol. Pharmacol.* **5,** 593-604.

Toda M. and Abi-Dargham A. (2007) Dopamine hypothesis of schizophrenia: making sense of it all. *Curr. Psychiatry Rep.* **9,** 329-336.

Toda N., Tokuyama T., Seno S., Hirata F. and Hayaishi O. (1974) Effects of 5-hydroxykynurenamine, a new serotonin metabolite, on isolated dog basilar arteries. *Proc. Natl. Acad. Sci. U. S. A.* **71,** 122-124.

Toda N. (1975) Analysis of the effect of 5-hydroxykynurenamine,, a serotonin metabolite, on isolated cerebral arteries, aortas and atria. *J. Pharmacol. Exp. Ther.* **193,** 385-392.

Traynor B. J., Bruijn L., Conwit R., Beal F., O'Neill G., Fagan S. C. and Cudkowicz M. E. (2006) Neuroprotective agents for clinical trials in ALS: a systematic assessment. *Neurology* **67,** 20-27.

Tribl F., Arzberger T., Riederer P. and Gerlach M. (2007) Tyrosinase is not detected in human catecholaminergic neurons by immunohistochemistry and Western blot analysis. *J. Neural Transm. Suppl.* 51-55.

Tsuda H., Noguchi T. and Kido R. (1972) 5-hydroxytryptophan pyrrolase in rat brain. *J. Neurochem.* **19,** 887-890.

Tsuda H., Noguchi T. and Kido R. (1974) Formamidase in rat brain. *J. Neurochem.* **22,** 679-683.

Turner E. H., Loftis J. M. and Blackwell A. D. (2006) Serotonin a la carte: supplementation with the serotonin precursor 5-hydroxytryptophan. *Pharmacol. Ther.* **109,** 325-338.

Twarog B. M., Page I. H. and Bailey H. (1953) Serotonin content of some mammalian tissues and urine and a method for its determination. *Am. J. Physiol.* **175,** 157-161.

Tzschentke T. M. (2001) Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog. Neurobiol.* **63,** 241-320.

Udenfriend S. and Cooper J. R. (1952) The enzymatic conversion of phenylalanine to tyrosine. *J. Biol. Chem.* **194,** 503-511.

van der Veen F. M., Evers E. A. T., Deutz N. E. P. and Schmitt J. A. J. (2007) Effects of acute tryptophan depletion on mood and facial emotion perception related brain activation and performance in healthy women with and without a family history of depression. *Neuropsychopharmacology* **32,** 216-224.

van Spronsen F. J., Hoeksma M. and Reijngoud D. J. (2009) Brain dysfunction in phenylketonuria: is phenylalanine toxicity the only possible cause? *J. Inherit. Metab. Dis.* **32,** 46-51.

Vassilacopoulou D., Sideris D. C., Vassiliou A. G. and Fragoulis E. G. (2004) Identification and characterization of a novel form of the human L-dopa decarboxylase mRNA. *Neurochem. Res.* **29,** 1817-1823.

Venugopal R. and Jaiswal A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14960-14965.

Verbeek M. M., Geurtz P. B. H., Willemsen M. A. A. P. and Wevers R. A. (2007) Aromatic L-amino acid decarboxylase enzyme activity in deficient patients and heterozygotes. *Mol. Genet. Metab.* **90,** 363-369.

Vichai V. and Kirtikara K. (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* **1,** 1112-1116.

Voltattorni C. B., Minelli A. and Dominici P. (1983) Interaction of aromatic amino acids in D-form and L-form with 3,4-dihydroxyphenylalanine decarboxylase from pig-kidney. *Biochemistry (Mosc).* **22,** 2249-2254.

von Ballmoos C., Cook G. M. and Dimroth P. (2008) Unique rotary ATP synthase and its biological diversity. *Annu. Rev. Biophys.* **37,** 43-64.

Wada H. and Snell E. E. (1961) The enzymatic oxidation of pyridoxine and pyridoxamine phosphates. *J. Biol. Chem.* **236,** 2089-2095.

Wade L. A. and Katzman R. (1975) 3-O-methyldopa uptake and inhibition of L-dopa at the blood-brain barrier. *Life Sci.* **17,** 131-136.

Wakamatsu K., Fujikawa K., Zucca F. A., Zecca L. and Ito S. (2003) The structure of neuromelanin as studied by chemical degradative methods. *J. Neurochem.* **86,** 1015-1023.

Walker J. E. (1998) ATP synthesis by rotary catalysis (Nobel Lecture). *Angewandte Chemie-International Edition* **37,** 2309-2319.

Wang X. J., Hayes J. D., Higgins L. G., Wolf C. R. and Dinkova-Kostova A. T. (2010b) Activation of the NRF2 signaling pathway by copper-mediated redox cycling of para- and ortho-hydroquinones. *Chem. Biol.* **17,** 75-85.

Wang Y., Lawson M. A., Dantzer R. and Kelley K. W. (2010a) LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-gamma-independent manner

by a JNK signaling pathway in primary murine microglia. *Brain Behav. Immun.* **24,** 201-209.

Wassenberg T., Willemsen M. A., Geurtz P. B., Lammens M., Verrijp K., Wilmer M., Lee W. T., Wevers R. A. and Verbeek M. M. (2010) Urinary dopamine in aromatic L-amino acid decarboxylase deficiency: The unsolved paradox. *Mol. Genet. Metab.*

Watts S. W., Gilbert L. and Webb R. C. (1995) 5-Hydroxytryptamine2B receptor mediates contraction in the mesenteric artery of mineralocorticoid hypertensive rats. *Hypertension* **26,** 1056-1059.

Weiner D. M., Levey A. I., Sunahara R. K., Niznik H. B., Odowd B. F., Seeman P. and Brann M. R. (1991) D1 and D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1859-1863.

Weissbach H., Redfield B. G. and Axelrod J. (1960) Biosynthesis of melatonin - enzymic conversion of serotonin to N-acetylserotonin. *Biochim. Biophys. Acta* **43,** 352-353.

Wharton D. C. and Tzagoloff A. (1967) Cytochrome oxidase from beef heart mitochondria. *Meth. Enzymol.* **10,** 245-253.

Whetsell W. O., Jr. and Schwarcz R. (1989) Prolonged exposure to submicromolar concentrations of quinolinic acid causes excitotoxic damage in organotypic cultures of rat corticostriatal system. *Neurosci. Lett.* **97,** 271-275.

Whyte M. P., Mahuren J. D., Vrabel L. A. and Coburn S. P. (1985) Markedly increased circulating pyridoxal-5'-phosphate levels in hypophosphatasia. Alkaline phosphatase acts in vitamin B6 metabolism. *J. Clin. Invest* **76,** 752-756.

Wikstrom M. K. (1977) Proton pump coupled to cytochrome c oxidase in mitochondria. *Nature* **266,** 271-273.

Wild A. C., Moinova H. R. and Mulcahy R. T. (1999) Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J. Biol. Chem.* **274,** 33627-33636.

Winder W. W. and Holloszy J. O. (1977) Response of mitochondria of different types of skeletal muscle to thyrotoxicosis. *Am. J. Physiol.* **232,** C180-C184.

Wise R. A., Spindler J., Dewit H. and Gerber G. J. (1978) Neuroleptic-induced anhedonia in rats - pimozide blocks reward quality of food. *Science* **201,** 262-264.

Wittig I., Braun H. P. and Schagger H. (2006) Blue native PAGE. *Nat. Protoc.* **1,** 418-428.

Wojcik S. M. and Brose N. (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: Speed and accuracy matter. *Neuron* **55,** 11-24.

Woolf L. I., Griffiths R. and Moncrieff A. (1955) Treatment of phenylketonuria with a diet low in phenylalanine. *Br. Med. J.* **1,** 57-64.

Wu E. Y., Smith M. T., Bellomo G. and Di Monte D. (1990) Relationships between the mitochondrial transmembrane potential, ATP concentration, and cytotoxicity in isolated rat hepatocytes. *Arch. Biochem. Biophys.* **282,** 358-362.

Wu K. L., Hsu C. and Chan J. Y. (2007) Impairment of the mitochondrial respiratory enzyme activity triggers sequential activation of apoptosis-inducing factor-dependent and caspase-dependent signaling pathways to induce apoptosis after spinal cord injury. *J. Neurochem.* **101,** 1552-1566.

Wullner U., Reimold M., Abele M., Burk K., Minnerop M., Dohmen B. M., Machulla H. J., Bares R. and Klockgether T. (2005) Dopamine transporter positron emission tomography in spinocerebellar ataxias type 1, 2, 3, and 6. *Arch. Neurol.* **62,** 1280-1285.

Wyatt R. J., Engelman K., Kupfer D. J., Scott J., Sjoerdsma A. and Snyder F. (1969) Effects of para-chlorophenylalanine on sleep in man. *Electroencephalogr. Clin. Neurophysiol.* **27,** 529-532.

Xie X. S. and Stone D. K. (1986) Isolation and reconstitution of the clathrin-coated vesicle proton translocating complex. *J. Biol. Chem.* **261,** 2492-2495.

Yamada J., Sugimoto Y., Wakita H. and Horisaka K. (1988) The involvement of serotonergic and dopaminergic systems in hypothermia induced in mice by intracerebroventricular injection of serotonin. *Jpn. J. Pharmacol.* **48,** 145-148.

Yoshida R., Imanishi J., Oku T., Kishida T. and Hayaishi O. (1981) Induction of pulmonary indoleamine 2,3-dioxygenase by interferon. *Proc. Natl. Acad. Sci. U. S. A.* **78,** 129-132.

Yoshida R., Oku T., Imanishi J., Kishida T. and Hayaishi O. (1986) Interferon: a mediator of indoleamine 2,3-dioxygenase induction by lipopolysaccharide, poly(I) X poly(C), and pokeweed mitogen in mouse lung. *Arch. Biochem. Biophys.* **249,** 596-604.

Yudkoff M., Daikhin Y., Nissim A., Horyn O., Luhovyy B., Lazarow A. and Nissim I. (2005) Brain amino acid requirements and toxicity: The example of leucine. *J. Nutr.* **135,** 1531S-1538S.

Zafar K. S., Inayat-Hussain S. H., Siegel D., Bao A., Shieh B. and Ross D. (2006) Overexpression of NQO1 protects human SK-N-MC neuroblastoma cells against dopamine-induced cell death. *Toxicol. Lett.* **166,** 261-267.

Zamboni N. and Sauer U. (2009) Novel biological insights through metabolomics and 13C-flux analysis. *Curr. Opin. Microbiol.* **12,** 553-558.

Zannoni V. G. and Weber W. W. (1966) Isolation and properties of aromatic alpha-keto acid reductase. *J. Biol. Chem.* **241,** 1340-1344.

Zawilska J. B., Skene D. J. and Arendt J. (2009) Physiology and pharmacology of melatonin in relation to biological rhythms. *Pharmacol. Rep.* **61,** 383-410.

Zeng B. Y., Pearce R. K., MacKenzie G. M. and Jenner P. (2001) Chronic high dose L-dopa treatment does not alter the levels of dopamine D-1, D-2 or D-3 receptor in the striatum of normal monkeys: an autoradiographic study. *J. Neural Transm.* **108,** 925-941.

Zhang Y., Marcillat O., Giulivi C., Ernster L. and Davies K. J. (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J. Biol. Chem.* **265,** 16330-16336.

Zhou F. C., Tao-Cheng J. H., Segu L., Patel T. and Wang Y. (1998) Serotonin transporters are located on the axons beyond the synaptic junctions: Anatomical and functional evidence. *Brain Res.* **805,** 241-254.

Zhu M. Y., Juorio A. V., Paterson I. A. and Boulton A. A. (1992) Regulation of aromatic L-amino acid decarboxylase by dopamine receptors in the rat brain. *J. Neurochem.* **58,** 636-641.

Zipper L. M. and Mulcahy R. T. (2002) The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J. Biol. Chem.* **277,** 36544-36552.

Zucker M. B. (1944) A study of the substances in blood serum and platelets which stimulate smooth muscle. *Am. J. Physiol.* **142,** 12-26.

8.1 APPENDIX 1: Publications

8.1.1 List of Published Journal Articles Related to this Thesis:

Allen G. F., Land J. M. and Heales S. J. (2009) A new perspective on the treatment of aromatic L-amino acid decarboxylase deficiency. *Mol. Genet. Metab.* **97,** 6-14.

Allen G. F., Neergheen V., Oppenheim M., Fitzgerald J. C., Footitt E., Hyland K., Clayton P. T., Land J. M. and Heales S. J. (2010) Pyridoxal 5'-phosphate deficiency causes a loss of aromatic L-amino acid decarboxylase in patients and human neuroblastoma cells, implications for aromatic L-amino acid decarboxylase and vitamin B6 deficiency states. *J. Neurochem.* **114,** 87-96.

Brun L., Ngu L. H., Keng W. T., Ch'ng G. S., Choy Y. S., Hwu W. L., Lee W. T., Willemsen M. A., Verbeek M. M., Wassenberg T., Regal L., Orcesi S., Tonduti D., Accorsi P., Testard H., Abdenur J. E., Tay S., Allen G. F., Heales S., Kern I., Kato M., Burlina A., Manegold C., Hoffmann G. F. and Blau N. (2010) Clinical and biochemical features of aromatic L-amino acid decarboxylase deficiency. *Neurology* **75,** 64-71.

8.1.2 List of Published Conference Abstracts Related to this Thesis:

Allen G., Clayton P., Land J., Hyland K. and Heales S. (2008) Pyridoxal phosphate availability and aromatic amino acid decarboxylase activity. Implications for AADC deficiency and inborn errors of vitamin B6 metabolism. *J. Inherit. Metab. Dis.* **31,** S138.

Allen G., Ullah Y., Land J. and Heales S. (2010) Dopamine may influence brain glutathione: implications for aromatic L-amino acid decarboxylase deficiency and other inherited conditions of dopamine metabolism. *J. Inherit. Metab. Dis.* **33,** S164.