Kynurenines in Neurological Disorders

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Abstract

The kynurenine pathway is thought to be involved in neurological disorders but its precise role and the mechanisms involved have yet to be established. Tryptophan can be metabolised via this pathway to produce the neurotoxic *N*-methyl-*D*-aspartate (NMDA) receptor agonist, quinolinic acid (QUIN), and the direct generators of reactive oxygen species, 3-hydroxykynurenine (3HKYN) and 3-hydroxyanthranilic acid (3HANA), as well as the neuroprotective NMDA receptor antagonist, kynurenic acid (KYNA).

High performance liquid chromatography (HPLC) methods were successfully developed and validated for measuring tryptophan, kynurenine, KYNA, 3HANA and anthranilic acid (ANA) in blood samples, using absorbance and fluorescence detection. The method for determining 3HKYN using electrochemical detection was more problematic and was only used for tryptophan loaded samples and their respective baseline samples.

Using HPLC, the concentrations of tryptophan, kynurenine, KYNA, 3HKYN and 3HANA were measured in the blood of Huntington's disease (HD) patients and patients with chronic brain injury, where the injury had occurred at least one year previously. QUIN was also determined for these patients using gas chromatography-mass spectrometry (GC-MS). In addition, the dynamics of the kynurenine pathway were investigated following oral tryptophan depletion and loading. In contrast to these chronic conditions, patients with acute stroke were also studied. The concentrations of tryptophan, kynurenine, KYNA, ANA and 3HANA were determined in the blood of the stroke patients, examining any changes in these concentrations during the two weeks after the stroke. The extent of inflammation and oxidative stress were also assessed for all patients, by measuring the levels of neopterin and the lipid peroxidation products, malondialdehyde and 4-hydroxynonenal.

Patients with late stage HD showed abnormal tryptophan metabolism via the kynurenine pathway, together with increased inflammation and oxidative stress. Increased levels of kynurenine together with increased kynurenine: tryptophan (K:T) ratios, indicating greater indoleamine 2,3-dioxygenase (IDO) activity, were observed in blood samples from HD patients in comparison with healthy control subjects. In conjunction with this increased IDO activity, there was a decrease in the ratios of KYNA: kynurenine, suggesting decreased kynurenine aminotransferase (KAT) activity. Inflammation, which may be stimulating IDO activity, could also be decreasing KAT activity, suggested by negative

correlations between the KYNA: kynurenine ratios and the inflammatory marker, neopterin. The inactivity of KAT suggests a small deficiency in KYNA over a long period of time which could cause a reduction in NMDA receptor antagonism, resulting in slow progressive excitotoxicity contributing to the neurodegeneration in HD. Low KYNA: kynurenine ratios were observed in baseline and tryptophan depleted samples, but after tryptophan loading, HD patients showed similar ratios compared with control subjects. This suggests that loading may be beneficial for HD patients, as more of the neuroprotectant, KYNA can potentially be produced. However, the results suggest that concentrations of the neurotoxin, QUIN, may also be increasing after tryptophan loading. Low concentrations of 3HKYN and 3HANA, with no change in QUIN levels, were also observed in the blood of HD patients. 3HANA levels continued to be decreased for the HD patients after loading. This may suggest degradation of 3HKYN and 3HANA by autoxidation producing reactive oxygen species which could contribute to the high levels of oxidative stress found in these patients.

Tryptophan loading in healthy control subjects showed significant increases in the inflammatory marker, neopterin, and in the lipid peroxidation products. These results should be considered when tryptophan loading is used in psychiatric practice and in diets high in tryptophan, such as the Atkins diet.

Patients with severe chronic brain injury showed similar alterations in kynurenine pathway metabolism as HD patients, at baseline and throughout the loading and depletion protocols. Although the brain injury had occurred at least one year previously, these patients showed persistent inflammation and oxidative stress, demonstrated by their increased levels of neopterin and lipid peroxidation products compared with healthy controls. In baseline blood samples, there were increased K:T ratios indicating greater IDO activity in the patients. Patients with chronic brain injury showed decreased concentrations of the neuroprotectant, KYNA, as well as low KAT activity, indicated by the decreased KYNA: kynurenine ratios. After tryptophan loading, K:T ratios decreased and the KYNA: kynurenine ratios increased in patients in comparison with controls, suggesting a reversal in the activities of the enzymes IDO and KAT. Similar levels of the inflammatory marker, neopterin, were observed in patients and controls after tryptophan loading. This suggests that these changes in IDO and KAT activities may be related to inflammation. As for the HD patients, patients with chronic brain injury showed lower levels of 3HKYN and 3HANA in their blood, with no change in QUIN levels. These metabolites may be undergoing autoxidation, producing reactive oxygen species which contribute to the ongoing oxidative stress in these patients.

The kynurenine pathway was activated following an acute stroke, as indicated by the increased K:T ratios, suggesting greater IDO activity. Stroke patients also had raised levels of neopterin and lipid peroxidation products, indicating inflammation and oxidative stress. There were no changes in the blood concentrations of kynurenines, neopterin or lipid peroxidation products during the fourteen days after a stroke. Stroke patients had reduced levels of 3HANA in their blood, as observed for the HD and chronic brain injury patients. There were negative correlations between the concentration of 3HANA and the volume of the brain lesion, measured by computed tomography (CT) scan, demonstrating the importance of the decreased concentrations of 3HANA. In addition, there were increased levels of ANA in the blood of the stroke patients and the ratios of 3HANA: ANA also correlated with brain lesion volume. Another measurement which correlated with lesion volume was lipid peroxidation, suggesting that oxidative stress contributes to the extent of the brain damage after a stroke. This may suggest that the role of 3HANA in stroke is related to its autoxidation and the generation of reactive oxygen species. Increased concentrations of KYNA were observed in patients who died within three weeks of having a stroke. These high levels of KYNA may have been produced following excitotoxicity and the generation of free radicals, and may cause excessive NMDA receptor blockade or reduced mitochondrial adenosine triphosphate (ATP) synthesis, thus contributing to cell death.

The kynurenine pathway was activated and showed abnormal metabolism in all the patient groups, suggesting a potential role for these metabolites in neuronal dysfunction in HD, chronic brain injury and acute stroke. Further work is required to elucidate the role of tryptophan metabolites and whether they may have a direct contribution to neuronal damage in neurological disorders.

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Papers

Mackay G.M., Forrest C.M., Stoy N., Christofides J., Egerton M., Stone T.W. and Darlington L.G. (2006) Tryptophan metabolism in patients with chronic brain injury. *Eur. J. Neurol.* **13,** 30-42 (2006).

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Papers in Preparation

Darlington L.G., **Mackay G.M.**, Forrest C.M., George C. and Stone T.W. Kynurenine metabolites of tryptophan may contribute to stroke-induced brain injury.

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Author's Declaration

I, Gillian Moira Mackay, declare that this thesis was composed by myself, and also that the experiments described therein were performed by myself, except where referenced.

Gillian M. Mackay
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Abbreviations

3HANA	3-hydroxyanthranilic acid
3HAO	3-hydroxyanthranilate 3,4-dioxygenase
3HKYN	3-hydroxykynurenine
5HT	5-hydroxytryptamine
8-OHDG	8-hydroxy-deoxyguanosine
ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANA	anthranilic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computed tomography
CuZn-SOD	copper, zinc-dependent superoxide dismutase
CV	coefficient of variation
DNA	deoxyribonucleic acid
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetate
ESR	erythrocyte sedimentation rate
GABA	γ-aminobutyric acid
GC-MS	gas chromatography – mass spectrometry
HD	Huntington's disease
HPLC	high performance liquid chromatography
HSP	heat shock protein
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-γ
IL	interleukin
iNOS	inducible nitric oxide synthase
IPA	indole-3-pyruvic acid
KAT	kynurenine aminotransferase
KMO	kynurenine 3-mono-oxygenase

K:T ratio	kynurenine: tryptophan ratio
KYNA	kynurenic acid
LDL	low density lipoprotein
LoD	limit of detection
MCAO	middle cerebral artery occlusion
mitAAT	mitochondrial aspartate aminotransferase
Mn-SOD	manganese-dependent superoxide dismutase
MRI	magnetic resonance imaging
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NGF	nerve growth factor
NMDA	*N*-methyl-*D*-aspartate
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
OGD	oxygen glucose deprivation
PARP	poly (ADP-ribose) polymerase
PCA	perchloric acid
PET	positron emission tomography
PBS	phosphate buffered saline
QPRT	quinolinate phosphoribosyltransferase
QUIN	quinolinic acid
ROS	reactive oxygen species
rt-PA	recombinant tissue plasminogen activator
SD	standard deviation
SEM	standard error of the mean
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SOD	superoxide dismutase
S-PBN	*N*-tert-butyl-α-(2-sulphophenyl)-nitrone
SPE	solid phase extraction
STAIR	Stroke Therapy Academic Industry Roundtable
STAZN	stilbazulenyl nitrone
TBI	traumatic brain injury
TDO	tryptophan 2,3-dioxygenase
TGF-β	transforming growth factor-β
TMPO	3,3,5,5-tetramethylpyrroline *N*-oxide
TNF-α	tumour necrosis factor-α

1 Introduction

1.1 Kynurenine Pathway

1.1.1 General

The first indication that kynurenines may have a role in brain function was the report by Lapin (1978), who observed convulsions in mice after an intracerebroventricular quinolinic acid (QUIN) injection. In 1981, Stone and Perkins discovered that QUIN was a potent excitant of neurones in the central nervous system (CNS), by acting as an agonist at the *N*-methyl-*D*-aspartate (NMDA) sensitive population of glutamate receptors. Intracerebrally injected QUIN was shown to produce excitotoxic lesions in the rat brain (Schwarcz *et al.*, 1983). Kynurenic acid (KYNA) was discovered to act as an antagonist at glutamate receptors (Perkins and Stone, 1982) and helps to prevent the brain damage caused by QUIN (Foster *et al*., 1984a). Other kynurenines, 3-hydroxykynurenine (3HKYN) and 3-hydroxyanthranilic acid (3HANA), can produce neuronal damage, largely by generating free radical species which cause oxidative stress (Eastman and Guilarte, 1989; Okuda *et al*., 1998). QUIN can also generate free radicals and cause oxidative cell damage (Rios and Santamaria, 1991). The major components of the kynurenine pathway are shown in Figure 1-1.

1.1.2 *N*-methyl-*D*-aspartate Receptor Activity

Excitatory amino acid-mediated synaptic transmission is the most prevalent excitatory system within the mammalian brain. Glutamate is the main excitatory neurotransmitter in the human CNS. Glutamate is a common amino acid used throughout the human body to synthesise proteins (Cooper *et al*., 2003). The arrival of action potentials at a synaptic terminal of neurones triggers the influx of Ca^{2+} ions across the cell membrane from the extracellular space into the neuronal terminal (Stone, 1995). Glutamate is stored in synaptic vesicles, and on depolarisation of the nerve terminal, it is released by calcium-dependent exocytosis into the synaptic cleft. Post-synaptic ionotropic receptors sensitive to NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate are activated. Glutamate's removal from the synaptic cleft is by a high affinity cellular uptake process, back into the pre-synaptic nerve terminal and/or into glial cells. Excessive stimulation of these receptors results in excitotoxicity. Neuronal excitotoxicity usually refers to the death of neurones arising from prolonged exposure to high concentrations of

glutamate and the excessive influx of ions and water into the cell. The resulting overload of calcium ions is particularly neurotoxic, leading to activation of enzymes that degrade proteins, membranes and deoxyribonucleic acid (DNA) (Fan and Raymond, 2007).

The synaptic activity of NMDA receptors under normal physiological conditions controls cognitive functions, such as learning and memory, as well as sensory and motor abilities (Ikonomidou and Turski, 2002). However, pathological activation of the NMDA receptors is thought to cause excitotoxic neuronal damage in ischaemia, epilepsy and Huntington's disease (HD) (Swartz *et al*., 1990). QUIN is an endogenous excitatory compound which acts as a weak agonist at NMDA receptors and has been suggested to contribute to this neuronal damage (Stone and Perkins, 1981; Tsuzuki *et al*., 1989).

Functional NMDA receptor complexes consist of a combination of NR1, NR2 and NR3 subunits. Molecular studies have identified various subtypes of each of these subunits, which result in a range of NMDA receptors, all with distinct distribution, properties and regulation. QUIN has been shown to activate some subtype combinations more than others (de Carvalho *et al*., 1996). The NR2 subunit contains the glutamate recognition site where the agonist binds, and the NR1 subunit contains the glycine recognition site (Parsons *et al*., 1998). NR3 subunits decrease the magnitude of NR1/NR2 receptor-mediated currents or form glycine-activated channels with the NR1 subunit alone (Wada *et al*., 2006). Glycine (or D-serine) is a co-agonist at NMDA receptors and its binding at the glycine site is necessary for receptor activation by the agonist. There is a cation binding site inside the channel where magnesium (Mg^{2+}) ions bind. These ions block conductance through the channel via voltage-dependent gating. When the glutamate binding site is occupied by an agonist, depolarisation results in removing the voltage-dependent Mg^{2+} block within the ion channel, leading to Ca^{2+} ion influx into the neurones. NMDA receptors have high permeability to Ca^{2+} ions (de Carvalho *et al*., 1996) and NMDA receptor-mediated neurotransmission occurs slowly with prolonged opening of the ion channel (Fan and Raymond, 2007). Polyamines, such as spermine and spermidine, are modulators which enhance the activation of NMDA receptors, although at high concentrations they can also block the receptors (Parsons *et al*., 1998; St'astny *et al*., 1999).

Excessive stimulation of NMDA receptors with glutamate or QUIN results in excitotoxicity and many studies have examined the potential role of antagonists at this receptor to offer neuroprotection. Competitive antagonists, often analogues of dicarboxylic amino acids, can inhibit NMDA receptor activation by competing with the agonist for binding to the glutamate recognition site (Foster and Fagg, 1987). There is

another binding site within the channel where non-competitive NMDA receptor antagonists such as dizocilpine (MK-801) block the NMDA channel and receptor activation (Foster and Fagg, 1987). For binding of such antagonists to occur, the agonist must already be bound to the receptor. A further type of antagonism for NMDA receptors is inhibition of the glycine binding site. KYNA is an endogenous antagonist at this site, but in healthy brain, its concentration is too low to affect NMDA receptor function. Its levels can be manipulated in the brain by drugs and increased levels have been shown to offer neuroprotection in ischaemic gerbils (Speciale *et al*., 1996). As well as many other synthetic antagonists, 7-chlorokynurenic acid has a much higher affinity for the glycine site than KYNA itself (Parsons *et al*., 1998).

1.1.3 Tryptophan and Indoleamine 2,3-dioxygenase

Historically, tryptophan metabolism via the kynurenine pathway was studied in the 1950s and 1960s in order to understand its contribution to the synthesis of nicotinamide adenine dinucleotide (NAD^+), a coenzyme involved in basic cellular processes (Wolf, 1974). Tryptophan is an essential amino acid in the diet being required for protein biosynthesis and is a component of dietary proteins, plentiful in foods such as chocolate, oats, bananas, milk, meat, turkey and peanuts. Tryptophan can be metabolised via another pathway to form serotonin (5-hydroxytryptamine, 5HT), an important neurotransmitter involved in many disorders including depression (Kohl and Sperner-Unterweger, 2007). Melatonin is a hormone produced from tryptophan via 5HT. It regulates circadian rhythms, with melatonin supplements being available for insomnia and jet lag, and is also a powerful antioxidant (Nowak and Zawilska, 1998). The kynurenine pathway is the major route for the metabolism of non-protein tryptophan in most tissues. This oxidative pathway is greatly upregulated by an inflammatory or immune response, as this activates one of the initial enzymes of the pathway, indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17), which converts tryptophan to kynurenine.

At least two different enzymes, tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) and IDO, catalyse the irreversible cleavage of the indole ring of L-tryptophan, leading to the formation of formylkynurenine, which is then rapidly metabolised into kynurenine. TDO is a haem-dependent enzyme almost exclusively expressed in liver cells, with a half-life of approximately two hours in mammals and specifically oxidises L-tryptophan (Schutz *et al*., 1972). The administration of the substrate L-tryptophan (or an analogue such as α-methyltryptophan) greatly induces its activity. TDO activity can also be induced by glucocorticoids, causing a decrease in blood tryptophan levels and an increase in levels of

kynurenine, as demonstrated with hydrocortisone in the perfused rat liver (Green *et al*., 1976). Tryptophan can be transported from the blood to the brain, and hence glucocorticoids may cause a depletion of tryptophan in the brain which may cause a significant decrease in brain 5HT synthesis (Joseph *et al*., 1976). TDO may therefore be responsible for depression found in patients chronically treated with glucocorticoids. One of the kynurenine pathway metabolites, 3HANA, can reduce the efficiency of TDO (Schutz *et al.*, 1972).

Hepatic TDO activity is generally considered as the major cause of tryptophan metabolism via the kynurenine pathway in the body, but in response to an immune stimulation with pokeweed mitogen, Saito *et al.* (1993a) demonstrated increases in the blood levels of the kynurenines, L-kynurenine and QUIN, although there was a decrease in TDO activity. These kynurenines were produced from tryptophan by increased activity of the enzyme IDO.

IDO is a haem-containing protein able to catalyse a reaction between tryptophan and the superoxide anion. With the exception of superoxide dismutase (SOD), IDO is the only enzyme known to require the superoxide anion radical as a substrate (Hayaishi *et al*., 1977). Due to its reaction with superoxide, the induction of IDO may be expected to be an antioxidant defense mechanism but the superoxide can easily reform. The superoxide anion reduces the inactive ferric form of IDO to an active ferrous form, but IDO is rapidly autoxidised back to the ferric form, resulting in the release of superoxide (Takikawa, 2005). In contrast to TDO specifically acting on L-tryptophan, IDO may open the indole ring of all the indoleamines: 5HT, tryptamine, melatonin, D-tryptophan, L- or D-5-hydroxytryptophan, as well as L-tryptophan. Whereas TDO is predominantly located in the liver, IDO is present in most mammalian organs, including the central nervous system, intestine, placenta, lung, blood mononuclear phagocytes, epididymis and endocrine system, but not in the liver or the kidneys. In contrast with tryptophan inducing TDO activity, increased tryptophan concentrations may inhibit IDO activity, as interferon-γ (IFN-γ) stimulation of IDO in human cells in culture was reduced by a 10-fold increase in tryptophan levels from 50μM to 500μM (Werner *et al*., 1988).

1.1.4 IDO Activity and the Immune System

IDO is strongly induced by IFN-γ and other pro-inflammatory cytokines, by viruses, by bacterial lipopolysaccharide and other signals derived from activated T cells, while anti-inflammatory cytokines, such as interleukin-4 (IL-4), or growth factors such as

transforming growth factor-β (TGF-β), suppress IDO activity (Pfefferkorn, 1984). This suggests that this enzyme is under tight immunological control and that TDO is involved in general metabolism while IDO is related to immunological functions. IDO is activated in a variety of inflammatory diseases affecting the brain, such as meningitis, hepatic encephalopathy, septicaemia and neurovirological disorders e.g. acquired immunodeficiency syndrome (AIDS) (Heyes *et al.*, 1993).

Munn *et al*. (1998) demonstrated a physiological role of IDO in the regulation of the immune system during pregnancy. Placental cells express IDO, metabolise tryptophan and suppress maternal T cell proliferation, whereby the embryo is protected against rejection. Inhibition of IDO with 1-methyltryptophan was shown to cause rapid T cell-induced rejection of all allogeneic embryos in pregnant mice (Munn *et al*., 1998). *In vitro*, macrophages, induced to express IDO in response to IFN-γ and other signals from activating T cells, inhibit T cell proliferation by rapidly consuming tryptophan (Mellor and Munn, 2004). It is interesting that an immune response stimulates IDO activity, which in turn, suppresses T cell activity, showing a fine balance between IDO and the immune system. To produce an enhanced immune response, IDO activity would need to be inhibited. There is currently much interest in the inhibition of IDO for the treatment of cancer, as increased IDO activity has been implicated in the immune escape of tumour cells for a broad spectrum of cancers. In a murine breast cancer model, inhibition of IDO has been shown to improve responses to cancer chemotherapy (Muller *et al*., 2005). Conversely, IDO activity may be useful in reducing the immune response in autoimmune diseases and following transplants. Indeed, Bauer *et al.* (2005) showed prolonged survival of skin allografts in rats after treatment with kynurenine and 3HANA, metabolites that would be produced from increased IDO activity.

IDO also has a role in antimicrobial defense. Hayaishi *et al*. (1984) proposed that when viruses, bacteria or parasites invade host tissues, interferons are produced by macrophages and superoxide is generated by granulocytes. IFN-γ then induces IDO and tryptophan is degraded and depleted. Further growth of the viruses, bacteria or parasites is thus inhibited as tryptophan is an essential amino acid. Pfefferkorn (1984) demonstrated that IFN-γ blocks the growth of Toxoplasmic gondii, a common parasite in humans and animals, by inducing IDO in the host cells to degrade tryptophan. This effect was reversed by tryptophan addition in a dose-dependent manner. Werner *et al*. (1987) showed that human macrophages degraded tryptophan by the induction of IDO by IFN-γ. This effect was also

demonstrated in cultures of human astrocytes, activated by IFN-γ, with IDO inhibiting the growth of Toxoplasmic gondii (Oberdorfer *et al*., 2003).

1.1.5 IDO and Nitric Oxide Synthase Related Activities

As well as the kynurenine pathway, the nitric oxide synthase (NOS) pathway is also activated in blood monocytes and macrophages after stimulation with bacterial lipopolysaccharide or endogenous IFN-γ. The pathways seem to be interrelated as nitric oxide inhibits IDO and the formation of the kynurenines while inhibition of NOS results in excessive production of kynurenines (Thomas *et al*., 1994). One compound of the kynurenine pathway, 3HANA, has been shown to inhibit NOS activity (Sekkai *et al*., 1997), while another, picolinic acid, induces its activity (Melillo *et al*., 1994). Human astrocytes stimulated with IFN-γ increased IDO activity which caused the inhibition of toxoplasmal or bacterial growth (Oberdorfer *et al*., 2003). However, when these human astrocytes were stimulated with a cytokine cocktail to also express inducible NOS (iNOS), there was no inhibition of toxoplasmal or bacterial growth, demonstrating that the presence of iNOS regulated the IDO activity in the astrocytes. Nitric oxide is thought to be important for neuronal function and plasticity in the CNS, and this interaction may be relevant for understanding the physiology and pathology associated with the kynurenine pathway.

1.1.6 Kynurenine

The conversion of L-tryptophan to L-kynurenine by IDO is the first and rate-limiting step for the kynurenine pathway in the brain (Takikawa, 2005). Infusion of kynurenine, but not tryptophan, into the rat brain using a microdialysis technique, increased striatal extracellular fluid concentrations of KYNA, demonstrating a low IDO activity in the healthy rat brain (Swartz *et al*., 1990). Joseph *et al*. (1978) and Gal and Sherman (1978) were the first to identify L-kynurenine in brain tissue. L-kynurenine is also present in the blood and peripheral organs, occurring in low micromolar concentrations. Kynurenine from the periphery can cross the blood-brain barrier into the brain (Joseph and Kadam, 1979). About half of brain kynurenine is produced from tryptophan within the CNS, the rest originating as peripheral kynurenine (Forrest *et al*., 2005). Stone and Connick (1985) showed that kynurenine does not directly modify neuronal electrical activity in rat brain slices or in isolated neurones. Kynurenine can be metabolised in mammalian brain to form three different products: KYNA, 3HKYN and anthranilic acid (ANA).

1.1.7 Kynurenine Aminotransferases and Kynurenic Acid

KYNA is a neuroprotective product of the kynurenine pathway and is present in the human brain (Moroni *et al*., 1988). In mammalian peripheral organs, several pyridoxal phosphate-dependent aminotransferases are able to catalyse the transamination of kynurenine to KYNA (Okuno and Kido, 1991). In the rat and human brain, there are two established isoforms of kynurenine aminotransferases (KATs), known as KAT I (EC 2.6.1.64) and KAT II (EC 2.6.1.7) (Guidetti *et al*., 1997). In addition, a third enzyme, mitochondrial aspartate aminotransferase (mitAAT, EC 2.6.1.1), has recently been identified which can also produce KYNA from kynurenine in the mammalian brain (Guidetti *et al*., 2007a). Km values for the action of KAT I and KAT II, partially purified from rat brain, on kynurenine have been reported at high micromolar range for KAT II and low millimolar range for KAT I, at physiological pH (Guidetti *et al*., 1997). The Km value for an enzyme is the concentration of substrate that permits half maximal rate of reaction. KAT I and II differ substantially with regard to their pH optimum and substrate specificity. KAT I is a soluble enzyme and has a pH optimum of 9.5-10, preferring pyruvate as a co-substrate and is potently inhibited by competing substrates, including glutamine and tryptophan. KAT II acts optimally at physiological pH and preferentially recognises L-kynurenine as a substrate. The KAT II enzyme has a slight preference for 2-oxoglutarate as the co-substrate and can also transaminate α-aminoadipate and tryptophan, but not glutamine. Quisqualate but not tryptophan caused inhibition of KAT II activity (Guidetti *et al*., 1997). The enzyme mitAAT has a pH optimum of 8.0 and a low capacity to transaminate glutamine or α-aminoadipate. This enzyme was inhibited by aspartate, glutamate and quisqualate (Guidetti *et al*., 2007a). In the human and rat brain, KAT II is primarily responsible for the formation of KYNA, as KAT I is potently inhibited by physiological concentrations of glutamine and other abundant amino acids (Yu *et al.*, 2004), and low levels of mitAAT are present. In contrast, the enzyme mitAAT played a major role in mouse brain (Guidetti *et al*., 2007a). KAT II activity showed regional differences in the rat brain, with the olfactory bulb having the highest level and the cerebellum having the lowest, whereas KAT I was similarly active in most brain areas.

KYNA has been shown to reduce NMDA-, QUIN-, kainate- and quisqualate-induced neuronal excitation, by acting as an antagonist at all three ionotropic glutamate receptors (NMDA, AMPA and kainate receptors) and blocking synaptic transmission in the cerebral cortex, hippocampus, striatum and spinal cord (Perkins and Stone, 1982; Ganong *et al*., 1983; Herrling, 1985; Perkins and Stone, 1985). KYNA can act at the NMDA receptor as

a competitive antagonist, shown to inhibit glutamate binding at concentrations of 100μM-1mM, but more potently, at concentrations of 10-100μM, binds at the glycine allosteric site on the NMDA receptor complex to antagonise receptor function (Moroni *et al*., 1988; Kessler *et al.*, 1989). As increased glycine can also enhance NMDA receptor activity, the site may not be saturated *in vivo*, implying that under physiological conditions, KYNA has the potential to modulate the glycine site (Danysz *et al*., 1989). However, concentrations of only 0.2-1.5μM KYNA are present in normal human brain tissue, much lower than the levels required to antagonise NMDA receptors (Wu *et al*., 2000), but human brain KYNA levels show marked regional differences (Turski *et al*., 1988). KYNA is also a potent inhibitor of the α7 nicotinic acetylcholine receptor (Hilmas *et al*., 2001; Stone, 2007), a property which is likely to contribute to KYNA's efficacy as an anti-excitotoxic agent. KYNA has been reported to be present in the thalamus, globus pallidus, hippocampus, parietal cortex and frontal cortex of the human post-mortem brain, with the highest concentration in the caudate nucleus and the lowest in the cerebellum (Turski *et al*., 1988). KYNA can readily enter the extracellular compartment of the brain (Swartz *et al*., 1990) and there is no evidence for enzymic degradation or cellular reuptake, indicating that removal from the brain is by passive efflux (Turski and Schwarcz, 1988). Cerebral KYNA levels and function can be regulated by either changes in the levels of its substrate, kynurenine, or the activity of KAT (Wu *et al*., 1992). The intracellular availability of kynurenine for KYNA synthesis is regulated by the large neutral amino acid transporter, which controls the uptake of kynurenine in astrocytes (Speciale *et al*., 1989a). KYNA can also be formed in the brain from the enol tautomer of indole-3-pyruvic acid (IPA), the process requiring the presence of oxygen free radicals (Bartolini *et al*., 2003).

The neuroprotective properties of KYNA have been demonstrated in several studies *in vivo*. KYNA was shown to block the excitotoxic action of QUIN on striatal cholinergic neurones and prevent the resulting seizures in rats (Foster *et al*., 1984a). The destruction of cholinergic neurones and impairments in memory-related tasks, following QUIN injections into the nucleus basalis magnocellularis in the rat, were prevented by co-injection of KYNA (Wirsching *et al*., 1989). Pretreatment with KYNA reduced infarct size and improved neurological outcome after focal cerebral ischaemia in rats (Germano *et al*., 1987).

KYNA, at increased levels, will act as an antagonist at NMDA receptors and can therefore modify excitotoxic vulnerability, neurophysiological activity and behaviour. Several studies have investigated the effect of increasing the concentration of KYNA by using a

kynurenine 3-mono-oxygenase (KMO) inhibitor, which blocks the production of the neurotoxic kynurenines, 3HKYN, 3HANA and QUIN (Cozzi *et al*., 1999; Carpenedo *et al*., 2002). It is therefore unclear whether the beneficial effects observed in these studies are from the increase in KYNA or the decrease in 3HKYN, 3HANA or QUIN. Transgenic mice with a genomic disruption of KAT II (Yu *et al*., 2004) have a transient reduction in cerebral KYNA levels present during the first month of life, but KYNA levels return to normal thereafter. These mice were used in a novel experiment to test the hypothesis that KYNA reduces the vulnerability of striatal neurones to QUIN-induced neurotoxicity (Sapko *et al*., 2006). At post-natal day 14, the mice showed a significant reduction in KYNA levels but normal levels of 3HKYN and QUIN. Intrastriatal injections of QUIN caused significantly larger lesions than in age-matched wild-type mice, demonstrating a neuroprotective effect of KYNA. Recently, the first selective KAT II inhibitor, (*S*)-4-(ethylsulfonyl)benzoylalanine, has been synthesised and shown to inhibit KAT II *in vitro* and *in vivo* (Pellicciari *et al*., 2006). Using microdialysis, this inhibitor decreased the levels of extracellular KYNA in the hippocampus of rats and future work with this inhibitor should be useful in elucidating the specific roles of KYNA in the brain.

Elevated levels of KYNA in the brain have resulted after the administration of an excitotoxic compound. QUIN-induced lesions in the rat striatum or hippocampus increased KAT II but not KAT I activity and increased KYNA levels (Wu *et al*., 1992; Guidetti *et al*., 1997; Ceresoli-Borroni *et al.*, 1999). Systemic administration of kainate, as a model for epilepsy, also increased KYNA in the brain and plasma of rats (Baran *et al*., 1995). KYNA production offers neuroprotection, acting as a defense response to the excitotoxic insults. As excitotoxicity generates free radicals, one possible mechanism for these increases in KYNA formation is from the generation of nitric oxide, as nitric oxide donors have been shown to increase the production of KYNA in cortical brain slices (Luchowski and Urbanska, 2007). However, other neurotoxins, 3HKYN and quisqualate suppress KAT II activity (Guidetti *et al*., 1997). 3HKYN may act by competing with kynurenine as the substrate for the KAT II enzyme, producing xanthurenic acid.

KYNA levels decrease as a result of energy deprivation in rat cortical tissue slices, which can be reversed by the addition of 2-oxoacids, a co-substrate for KAT enzymes (Hodgkins and Schwarcz, 1998). However, high concentrations of greater than 125μM KYNA also lower the efficacy of heart mitochondrial adenosine triphosphate (ATP) synthesis, by activating oxygen consumption and by lowering ATP formation (Baran *et al*., 2003). The other kynurenines, ANA, 3HKYN and 3HANA can also affect heart mitochondria respiratory parameters, but concentrations need to be extremely high, above 1mM.

Tryptophan, kynurenine and QUIN showed no effect. Disturbances in mitochondrial function have been associated with neurodegenerative diseases (Beal, 1996), which could be attributed to these active kynurenines, especially KYNA. However, the high concentration of over 125μM KYNA needed to alter mitochondrial function is far in excess of that found in brain tissue (0.2-1.5μM) (Wu *et al*., 2000) and therefore this effect is unlikely to be relevant *in vivo*. 3HKYN and 3HANA probably have a more active role in impairing mitochondrial function by generating reactive oxygen species, which occurs at much lower concentrations (Okuda *et al*., 1996) than their effects on respiratory parameters. Another detrimental effect of increased levels of KYNA is its potential role as a contributory factor in the cognitive impairment in schizophrenia, due to it causing a reduction in glutamate receptor function (Schwarcz *et al*., 2001).

1.1.8 Kynureninase and Anthranilic Acid

A second enzyme, kynureninase (EC 3.7.1.3), can metabolise kynurenine into ANA and is also responsible for converting 3HKYN to 3HANA. As well as the KAT enzymes, kynureninase also requires pyridoxal phosphate (vitamin B6) and is especially sensitive to B6 deficiency. Elderly subjects after a tryptophan load showed a build up of kynurenine and 3HKYN in their plasma and urine, which was decreased by administering vitamin B6 (Crepaldi *et al*., 1975). 3HKYN levels were also markedly increased in the brain tissue of vitamin B6-deficient neonatal rats (Guilarte and Wagner, 1987).

ANA is not involved in the major route of the kynurenine pathway metabolism to produce QUIN, as kynureninase has much lower activity than KMO, the enzyme responsible for converting kynurenine to 3HKYN (Bender and McCreanor, 1982). However, following kynurenine administration, ANA is produced in the brain, and therefore alterations in kynurenine levels will also affect ANA concentrations (Chiarugi *et al*., 1996).

Although ANA is generally accepted to be biologically inactive, it can interact with copper to form an anti-inflammatory complex. This complex acts as a hydroxyl radical-inactivating ligand, able to remove the highly injurious hydroxyl radicals at inflammatory sites (Miche *et al*., 1997; Gaubert *et al*., 2000). The anthranilic acid-Cu^{2+} complex increases the Fenton reactivity of copper, producing more hydroxyl radicals, but quickly reacts with these radicals to render them inactive, with the overall effect being the removal of the hydroxyl radicals. Due to this property, the synthetic derivative of ANA, 3-methoxyanthranilate, has been proposed as a potential anti-inflammatory drug (Halova-Lajoie *et al*., 2006).

1.1.9 Kynurenine 3-mono-oxygenase and 3-Hydroxykynurenine

The third and main enzyme for kynurenine metabolism, kynurenine 3-mono-oxygenase (KMO, kynurenine 3-hydroxylase, E.C.1.14.13.9) metabolises kynurenine to 3HKYN. This enzyme has a high affinity for the substrate (Km in the low micromolar range), suggesting that, under physiological conditions, it metabolises most of the available kynurenine (Bender and McCreanor, 1982). KMO is localised in the outer mitochondrial membranes of brain, liver, placenta, spleen and kidney (Erickson *et al*., 1992). Saito *et al*. (1993c) reported that KMO expression increases under inflammatory conditions, demonstrated in the ischaemic gerbil brain.

3HKYN can be converted to xanthurenic acid by the aminotransferases, KAT I and KAT II, but these enzymes preferentially convert kynurenine to KYNA in the brain (Guidetti *et al*., 1997).

1.1.10 3-Hydroxyanthranilic Acid

3HKYN can be metabolised to form 3HANA by the enzyme, kynureninase. It is generally accepted that L-kynurenine is converted to QUIN via 3HKYN and 3HANA (Bender and McCreanor, 1982). In the mouse brain, Chiarugi *et al.* (1996) showed that 3HANA is predominantly produced by 3HKYN. In blood, ANA may produce more 3HANA, suggested to be due to microsomal hydroxylation of ANA that occurs in the liver (Ueda *et al*., 1978). In macrophage cultures, no labelled QUIN was formed in medium containing labelled ANA, but QUIN was produced in medium containing labelled tryptophan (Saito *et al*., 1993a). However, exposure of rat cortical brain slices to high concentrations of ANA resulted in the rapid production of 3HANA by direct hydroxylation (Baran and Schwarcz, 1990). ANA produced an 11-fold higher level of 3HANA compared with the 3HANA produced when 3HKYN was added to the brain slices. Biosynthesis of 3HANA from ANA cannot be excluded and may have a functional role in the brain.

3HANA can also contribute to reduced activity of the immune system, by inhibiting proliferation of T cells. Bauer *et al*. (2005) showed that kynurenine, 3HKYN and 3HANA strongly suppressed T cell proliferation, by causing T cell death, with immune-stimulated rat lymphocytes *in vitro*. Similarly, kynurenine, 3HKYN and 3HANA suppressed the T cell response to an allogeneic stimulus in dendritic cells, and the suppressive effects of these compounds were additive (Terness *et al*., 2002). Selective T cell apoptosis was demonstrated by 3HANA *in vitro* and *in vivo* (Fallarino *et al*., 2002). A consequence of

this reduced immune response mechanism was shown in rats where a mixture of kynurenine and 3HANA prolonged the survival of a skin allograft (Bauer *et al*., 2005).

1.1.11 Toxicity of 3-Hydroxykynurenine and 3-Hydroxyanthranilic Acid by Oxidative Stress

3HKYN and 3HANA can initially act as antioxidants as they undergo oxidation. However, these oxidation reactions generate potent oxidising agents *in vivo*, and thus contribute to oxidative stress. The redox behaviour of these compounds is dependent on the presence of other redox agents (Giles *et al*., 2003).

3HKYN levels were increased in the post-mortem brains of humans with neurological disorders, such as HD (Pearson and Reynolds, 1992), the AIDS-dementia complex (Sardar *et al*., 1995) and hepatic encephalopathy (Pearson and Reynolds, 1991). The brain concentrations of 3HKYN under these pathological conditions are estimated to be 0.3-1.2μM, whereas in non-diseased brains, 3HKYN levels are 0.08-0.3μM.

Intracerebroventricular administration of 3HKYN has been shown to be toxic in animals, and causes seizures (Lapin, 1981). Nakagami *et al*. (1996) demonstrated that intrastriatal injection in rats of 50nmoles 3HKYN induced tissue damage around the injection site. The antioxidant, *N*-acetyl-L-cysteine, reduced the 3HKYN damage, but the NMDA receptor inhibitor, MK-801, failed to protect the brain from 3HKYN toxicity. This suggests that oxidative stress and not the metabolism of 3HKYN into QUIN is causing the toxicity.

Jhamandas *et al.* (1990) showed that a single injection of 3HANA into the nucleus basalis magnocellularis in rats produced a large decrease in the survival of cholinergic neurones. The cause of the toxicity of 3HANA was explained as either the conversion of 3HANA into QUIN or the autoxidation of 3HANA producing reactive oxygen species.

At high concentrations, 3HKYN can be toxic to neurones by generating free radicals (Eastman and Guilarte, 1989). 3HKYN and 3HANA were toxic to primary neuronal cultures, with 3HANA being much less potent than 3HKYN (Okuda *et al*., 1998). Other metabolites of the kynurenine pathway, including kynurenine and ANA, were not toxic, suggesting that it is the *o*-aminophenol structure, common to 3HKYN and 3HANA, which is important for toxicity. *o*-Aminophenol compounds readily undergo several steps of oxidation reactions initiated by their oxidative conversion to quinoneimines, which are highly reactive and continue to oxidise. During these oxidation processes, various reactive

oxygen species are produced. The products of 3HKYN autoxidation, including hydrogen peroxide and xanthommatin, contribute to oxidative stress (Hiraku *et al*., 1995; Vazquez *et al*., 2000).

3HANA autoxidation has been suggested to involve 2 steps: the first producing the quinoneimine and converting molecular oxygen to the superoxide anion, the second condensation and oxidation reactions requiring molecular oxygen to form cinnabarinic acid and hydrogen peroxide (Dykens *et al*., 1987; Hiraku *et al.,* 1995). Under hypoxic treatment, there was no autoxidation of 3HANA, demonstrating that the reaction requires oxygen (Dykens *et al*., 1987).

The potency of 3HKYN and 3HANA is a quarter to a third of the oxidising capacity of hydrogen peroxide, but considerably stronger than glutathione disulfide, the common cellular oxidant (Giles *et al*., 2003).

In primary neuronal cultures from the rat striatum, 1μM 3HKYN caused toxicity resulting in approximately 50% cell death after 48h, with increased toxicity at 10μM and 100% cell death at 100μM but no toxicity at 0.1μM (Okuda *et al*., 1996). 3HKYN concentrations of 1μM were comparable to those found in diseased human post-mortem brains. Cultures from the striatum were chosen as this is a brain region where there is marked neuronal loss in HD. 3HANA required a concentration of greater than 10μM for 48h to demonstrate cell death (Okuda *et al.,* 1998). Cell lysis was shown to be a delayed effect, using hybrid neuronal cells in culture (Eastman and Guilarte, 1989). Following 2 hours exposure to 500μM 3HKYN, little cell lysis occurred, but removing the 3HKYN exposure and continuing to incubate the cells in fresh medium resulted in greater than 85% cell death after 8-12 hours.

Transition metal ions are required for 3HKYN and 3HANA toxicity, as iron chelation with desferrioxamine was able to inhibit 3HKYN-induced cell lysis (Eastman and Guilarte, 1990; Okuda *et al*., 1996). Iron ions can contribute to oxidative damage either by catalysing the autoxidation of 3HKYN or enabling the reduction of hydrogen peroxide to form the highly reactive hydroxyl radical, able to cause damage to macromolecules, such as DNA, proteins and lipid membranes. Goldstein *et al.* (2000) showed that 3HKYN and 3HANA in phosphate buffered saline (PBS) solution can potently reduce copper (Cu^{2+} ions forming Cu^{+}) and to a lesser extent iron (Fe^{3+} ions forming Fe^{2+}) at physiological pH. Other kynurenines, including kynurenine, ANA, and QUIN, were unable to reduce these transition metal ions. Even in the presence of trace metals in the PBS, 10μM 3HKYN and

3HANA could generate superoxide, but not hydrogen peroxide. When the metal chelator, diethylenetriaminepentaacetic acid (DTPA), was added, neither 3HKYN nor 3HANA could produce superoxide, indicating that superoxide generation by 3HKYN and 3HANA are metal-dependent reactions. 3HKYN and 3HANA can generate hydrogen peroxide in the presence of Cu^{2+} ions. Both 3HKYN and 3HANA generated approximately 3-fold more hydrogen peroxide than was expected from the available copper. It was suggested that copper may act as a catalyst, cycling between the reduced state produced by its reaction with 3HKYN, and the oxidised state produced by reacting with molecular oxygen.

In biological systems, most redox-active metals are bound to protein and small biomolecules. Whilst these metal ions are not free, they could still catalyse some reactions similar to those observed with the free ions. With inflammation or infection, local acidosis occurs, which liberates copper and iron from proteins, so they are more available for redox reactions. As inflammation or infection also stimulates the activity of the kynurenine pathway by activating IDO, increased levels of 3HKYN and 3HANA will be generated. Together, the free metal ions and the increased 3HKYN and 3HANA concentrations may well increase the production of reactive oxygen species such as superoxide and hydrogen peroxide (Goldstein *et al*., 2000).

Several features of apoptosis were observed in 3HKYN-mediated neuronal cell death (Okuda *et al*., 1998). Apoptosis is the mechanism of cell death known to often occur where cell death is induced by reactive oxygen species and has been implicated as the mechanism of neuronal damage in neurodegenerative diseases such as HD (Portera-Cailliau *et al.*, 1995). Wei *et al*. (2000) demonstrated that 3HKYN induced apoptosis in cerebellar granule cells and neuronally-related cell lines at high concentrations (>200μM), mainly by producing hydrogen peroxide, thought to be formed by autoxidation of 3HKYN in extracellular compartments. More evidence for a role of 3HKYN in neuronal cell death was shown by Jeong *et al*. (2004) where increased reactive oxygen species and increased caspase activity were involved in 3HKYN-induced apoptosis in a human neuroblastoma cell line. Lee *et al*. (2001) demonstrated 3HKYN-induced apoptosis in neuroblastoma cells and identified protection from this toxicity by heat shock proteins (HSP), in particular, HSP90, which inhibits caspases. Lee *et al*. (2004) showed that 3HKYN-induced neuronal cell death is associated with mitochondrial dysfunction and caspase activity. Following stimulation of IDO with IFN-γ, apoptosis was demonstrated in monocyte-derived cells in culture (Morita *et al*., 2001). 3HANA, but not 3HKYN or QUIN, induced apoptosis in these cells, and apoptosis was enhanced by the presence of redox-active metals ions.

The toxic effects of 3HKYN were abolished in the presence of many antioxidants, including catalase, glutathione, α-tocopherol, *N*-acetylcysteine and spin trapping agent, 3,3,5,5-tetramethylpyrroline *N*-oxide (TMPO), indicating that oxidative stress, particularly resulting from the production of hydrogen peroxide and the hydroxyl radical, is involved in 3HKYN toxicity (Eastman and Guilarte, 1989; Okuda *et al*., 1996; Okuda *et al*., 1998). It has been proposed that 3HKYN and 3HANA do promote the hydroxyl radical formation but simultaneously act as a site for hydroxyl radical attack, due to their initial antioxidant properties (Goldstein *et al*., 2000). This suggests that continued oxidation of 3HKYN or 3HANA occurs due to reaction with the hydroxyl radical. 3HKYN has been shown to scavenge hydroxyl radicals (Leipnitz *et al*., 2007).

The antioxidant enzyme, SOD, was not capable of reducing 3HKYN toxicity in neuronal cell cultures, which suggests that superoxide is not involved in cell lysis (Eastman and Guilarte, 1989; Okuda *et al*., 1996; Okuda *et al*., 1998). The superoxide anions formed from 3HANA autoxidation may be removed by SOD or transition metal cations. By removing this product of the reaction, the reverse reaction would be prevented and would result in accelerated 3HANA autoxidation (Dykens *et al*., 1987; Ishii *et al*., 1990). A similar mechanism may occur for 3HKYN. With an inflammatory or immune stimulus, IDO activity is increased and this is expected to increase the concentrations of 3HKYN and 3HANA. IDO uses superoxide as a substrate and hence its removal of superoxide may promote the autoxidation of 3HKYN and 3HANA (Goldstein *et al*., 2000).

Xanthine oxidase is also involved in 3HKYN toxicity as the addition of allopurinol, an inhibitor of xanthine oxidase, offered significant protection against 3HKYN toxicity (Okuda *et al*., 1996). Xanthine oxidase may catalyse the oxidation of 3HKYN to produce hydrogen peroxide. Alternatively, low concentrations of 3HKYN may stimulate xanthine oxidase activity, producing more free radicals from other oxidative reactions catalysed by this enzyme.

3HKYN toxicity from the accumulation of hydrogen peroxide has been demonstrated to occur intracellularly, with catalase and desferrioxamine able to act intracellularly to prevent toxicity (Eastman and Guilarte, 1990) and catalase and allopurinol both able to reduce intracellular hydrogen peroxide (Okuda *et al*., 1996). Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-containing neurones were spared from 3HKYN toxicity in cell culture, as they are in HD. NADPH diaphorase is contained in the specific aspiny neuronal subpopulation, co-localised with somatostatin and neuropeptide

Y. These neurones may have the ability to detoxify oxygen free radicals and are generally resistant to oxidative stress (Okuda *et al*., 1996).

Different brain regions may demonstrate different susceptibility to 3HKYN toxicity. In culture, cortical and striatal neurones were extremely vulnerable to 3HKYN toxicity, with hippocampal neurones being less susceptible and cerebellar neurones resistant to 3HKYN toxicity (Okuda *et al.*, 1998). Neither 3HANA nor hydrogen peroxide toxicity showed this regional selectivity. Extracellular 3HKYN can be taken up by cells either by sodium-independent or sodium-dependent cellular uptake mechanisms (Eastman *et al*., 1992), but there was a predominant role for the sodium-dependent process for the uptake of 3HKYN into striatal neurones (Okuda *et al.*, 1998). Competitive inhibition of 3HKYN uptake by an excess amount of another substrate for these transport systems, such as tryptophan, blocked 3HKYN neurotoxicity (Okuda *et al.*, 1998). This suggests that the differences in susceptibility to 3HKYN toxicity of the neurones from different brain regions are due to a difference in transporter activities of these neurones. Cerebellar neurones, with little sensitivity to 3HKYN toxicity, have poor ability to take up large neutral amino acids compared with striatal neurones with high sensitivity to 3HKYN toxicity. This evidence was supported by Speciale and Schwarcz (1990) who showed that sodium-dependent kynurenine uptake activity was greatest in the cortex, followed by the striatum, then by the hippocampus, which were all much greater than uptake in the cerebellum.

To counteract oxidative stress *in vivo*, there are several antioxidant defense mechanisms which consist of enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase), transition metal binding proteins and small molecular weight antioxidants (e.g. ascorbic acid, α-tocopherol). Potential therapies to reduce oxidative stress induced by 3HKYN or 3HANA include the chelation of redox-active metal ions or displacement with non-redox-active metal ions (Goldstein *et al*., 2000).

1.1.12 Antioxidant roles for 3-Hydroxykynurenine and 3-Hydroxyanthranilic Acid

In contrast, 3HKYN and 3HANA have been shown to exhibit antioxidant properties. Christen *et al*. (1990) showed that low micromolar concentrations of 3HKYN and 3HANA scavenged peroxyl radicals with high efficiency, being more protective than equimolar concentrations of ascorbate or Trolox (water soluble analogue of Vitamin E). IFN-γ induced kynurenine pathway metabolism in human peripheral blood mononuclear cells and

monocyte-derived macrophages, with physiological levels of tryptophan added. This resulted in increased levels of 3HANA, which could inhibit low density lipoprotein (LDL) oxidation when LDL was added (Christen *et al.*, 1994). Further work (Thomas *et al*., 1996) demonstrated that 3HANA inhibited peroxidation of LDL and proposed that 3HANA is a localised extracellular antioxidant defense against LDL oxidation in inflammation. Leipnitz *et al*. (2007) showed that the addition of 3HKYN and 3HANA to rat cerebral cortex, *in vitro*, and C6 glioma cells demonstrated antioxidant properties. Both compounds prevented lipid peroxidation and reduced peroxyl radicals. 3HANA prevented spontaneous glutathione oxidation in the cerebral cortex. 3HKYN could scavenge hydoxyl radicals. 3HANA can also have an anti-inflammatory protective effect by inducing haem oxygenase-1, which could offer protection in vascular injury such as atherosclerosis (Pae *et al*., 2006).

1.1.13 Quinolinic Acid

3HANA is readily converted to QUIN by the enzyme, 3-hydroxyanthranilate 3,4-dioxygenase (3HAO, EC 1.13.11.6) (Foster *et al*., 1986). 3HAO catalyses 3HANA into an intermediate product, aminocarboxymuconic semialdehyde, which then preferentially converts to QUIN by a non-enzymic cyclisation. This intermediate product can also produce picolinic acid instead of QUIN (Forrest *et al*., 2005).

Wolfensberger *et al.* (1983) and Moroni *et al.* (1984) demonstrated the presence of QUIN in the rat and human brain. The endogenous concentration of QUIN in healthy mammalian brain tissue has been reported as being 50-100nM and in human cerebrospinal fluid (CSF) has been shown to be 10-35nM (Heyes *et al*., 1992a; Moroni, 1999). For patients with inflammatory diseases, including various types of infections, meningitis, autoimmune diseases and septicaemia, increased levels of QUIN have been shown in both CSF and post-mortem brain tissue (Heyes *et al*., 1992a). In patients affected by the AIDS-dementia complex, the increase in QUIN levels in the CSF were over 20-fold and correlated with the severity of cognitive and motor disorders (Heyes *et a*l., 1991a). After traumatic brain injury (TBI), QUIN concentrations in the CSF were increased to between 5 and 50-fold, being higher in the patients who subsequently died (Sinz *et al*., 1998). QUIN accumulation occurs in the brains of experimental animal models of inflammatory neurologic diseases such as poliovirus brain infection (Heyes *et al*., 1992b), cerebral malaria (Sanni *et al*., 1998) and ischaemic brain disease (Saito *et al*., 1993b).

In 1981, Stone and Perkins discovered that QUIN was a potent excitant of cortical neurones in the rat brain by acting as an agonist at NMDA receptors. Administration of QUIN directly into the rat striatum, produced "axon-sparing" lesions, with marked swelling of dendrites and loss of cell structure in postsynaptic sites, but generally good preservation of axons and presynaptic terminals (Schwarcz *et al*., 1983; McGeer and Singh, 1984). No loss in glial cells was observed. Doses as low as 12nmoles QUIN caused neuronal loss around the injection site in the striatum (Schwarcz *et al*., 1983). Areas of the brain most sensitive to QUIN neurotoxicity are the hippocampus and striatum (Schwarcz and Kohler, 1983). Within these brain areas, some neuronal cell types are more sensitive than others, with cholinergic neuronal death in the striatum observed following QUIN injection (Foster *et al*., 1983) and preferential susceptibility of pyramidal cells in the hippocampus (Schwarcz *et al*., 1983). Striatal spiny neurones containing the neurotransmitter γ-aminobutyric acid (GABA) and substance P are also sensitive to QUIN toxicity, with the subclass of striatal aspiny neurones containing somatostatin and neuropeptide Y being preserved (Beal *et al*., 1986). QUIN acts selectively at NMDA receptors, specifically with NMDA receptor subtypes containing the NR2A and NR2B subunits (de Carvalho *et al*., 1996), with massive calcium entry into neurones, and therefore it is where these receptor subtypes are present that QUIN exerts the greatest damage to neurones. In rats, intrastriatal injection of QUIN resulted in an initial period of involuntary movements and intrahippocampal injection triggered convulsions (Foster *et al*., 1983).

The enzyme, quinolinate phosphoribosyltransferase (QPRT, EC 2.4.2.19) metabolises QUIN to form nicotinic acid ribonucleotide, which can then be metabolised to NAD^+. QPRT is present primarily in glial cells but is often found in cytoplasmic bodies separated from the rest of the cell, which may prevent the enzyme from accessing and metabolising QUIN as it is formed (Stone and Darlington, 2002). There are substantially fewer cells that contain QPRT than contain 3HAO (Kohler *et al*., 1988), suggesting that after stimulation of the kynurenine pathway, QUIN could accumulate.

Excitotoxicity induced by QUIN causes overactivation of NMDA receptors resulting in excessive Ca^{2+} ions entering the neurones. Mitochondrial dysfunction was shown by the reduction in mitochondrial oxygen consumption and a reduction in ATP levels following QUIN injection into the rat striatum (Bordelon *et al*., 1997). This could be caused by calcium loading of the mitochondria, which increases the production of free radicals. Mitochondrial dysfunction may be a critical event in the cell death cascade of QUIN excitotoxicity (Bordelon *et al*., 1997).

QUIN has been shown to cause neuronal death in human foetal brain tissue in culture at concentrations of 5 and 10mM, examined after 20 hours exposure (Kerr *et al*., 1995). Increases in the levels of QUIN have been observed for several inflammatory neurological disorders, but levels in the CSF (low micromolar) are still lower than those able to cause neuronal death *in vitro* (Heyes *et al*., 1992a). However, these effects may still be relevant as different neuronal populations may be selectively affected by QUIN, or alternatively, the neurotoxic potency of low concentrations of QUIN may increase during chronic exposure. Chiarugi *et al*. (2001) demonstrated that the neurotoxic threshold concentration of QUIN can be decreased from 100μM to 1μM by prolonging the time of exposure from 24 to 72 hours in primary cultures of mixed cortical cells (containing both neurones and glial cells). Excitotoxic damage was also shown in cultures of a rat corticostriatal system, but not in cultures of caudate nucleus, exposed to 100nM QUIN for 7 weeks (Whetsell and Schwarcz, 1989).

QUIN has a high *in vivo* potency as an excitotoxin, which cannot be fully explained by its activation of NMDA receptors (Foster *et al*., 1983). This may be caused by a high local concentration of QUIN at the synapse (Schwarcz, 1993) or the absence of effective removal mechanisms for extracellular QUIN (Foster *et al*., 1984b). However, it is likely that an additional mechanism is also involved (Foster *et al.,* 1983) as QUIN is able to readily generate damage-producing free radicals. High concentrations of QUIN (20-80μM) induced an increase in lipid peroxidation of up to 50% in rat brain homogenates after 30 minutes (Rios and Santamaria, 1991) and *in vivo*, intrastriatal injection of QUIN in the rat increased lipid peroxidation and caused neuronal damage (Santamaria and Rios, 1993).

The potent antioxidant, melatonin, when co-injected with QUIN into the rat hippocampus, inhibited the formation of lipid peroxidation products (Behan *et al*., 1999). Similarly, deprenyl, which scavenges free radicals and/or stimulates antioxidant enzymes, also prevented QUIN-induced lipid peroxidation in the rat hippocampus. Following QUIN intrastriatal injections in rats, a reduction in the level of the antioxidant glutathione, with simultaneous increases in its product glutathione disulphide, was shown (Rodriguez-Martinez *et al*., 2000). There was no change in glutathione peroxidase, which demonstrated a non-enzymatic mechanism of conversion of this endogenous antioxidant. Leipnitz *et al*. (2005) confirmed that QUIN reduced the brain non-enzymatic antioxidant defenses in rat brain homogenates *in vitro*. In addition, Rodriguez-Martinez *et al*. (2000) showed that cytosolic copper, zinc-dependent superoxide dismutase (CuZn-SOD) activity decreased after QUIN intrastriatal injection but mitochondrial manganese-dependent

superoxide dismutase (Mn-SOD) was not affected, suggesting that the intrastriatal toxicity of QUIN involves cytoplasmic oxidative injury. The antioxidant enzymes, CuZn-SOD and Mn-SOD can both reduce the concentrations of superoxide anions and prevent cell damage.

Stipek *et al*. (1997) showed that iron (as Fe^{2+} ions) is necessary for QUIN to demonstrate increased lipid peroxidation in rat brain homogenates, with the iron chelator, deferoxamine, completely preventing this effect. QUIN concentrations of 0.15 - 2.5mM stimulated lipid peroxidation over a range of concentrations of Fe^{2+} ions. Concentrations higher than 3mM inhibited lipid peroxidation, but such levels are unlikely to be reached *in vivo*. QUIN chelates Fe^{2+} ions but not Fe^{3+} ions, forming a QUIN-Fe^{2+} complex (Stipek *et al*., 1997; Iwahashi *et al*., 1999). It is interesting that the addition of Fe^{2+} ions increased lipid peroxidation in rat brain homogenates, without any QUIN present (Zaleska and Floyd, 1985). Fe^{2+} ions themselves induce lipid peroxidation as they catalyse the production of the hydroxyl radical from hydrogen peroxide via the Fenton reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \qquad \text{FENTON REACTION}$$

The Fe^{3+} ions produced can be recycled back to Fe^{2+} ions by reacting with more hydrogen peroxide. Both iron salts and hydrogen peroxide are available *in vivo* for the Fenton reaction. QUIN may act like other iron chelators, such as ethylenediaminetetraacetate (EDTA), which can also either stimulate or inhibit iron-dependent lipid peroxidation, depending on their concentration (Halliwell and Gutteridge, 1984). In phosphate buffer, the QUIN-Fe^{2+} chelate enhanced the formation of the hydroxyl radical via the Fenton reaction compared with Fe^{2+} ions alone (Iwahashi *et al*., 1999). Further investigation of the mechanism has suggested that the QUIN-Fe^{2+} chelate is relatively stable at physiological pH, with slow autoxidation, and although this initiates the generation of hydroxyl radicals via the Fenton reaction, a further QUIN derivative is formed, which enables redox cycling of Fe^{2+} and Fe^{3+} ions, thus maintaining hydroxyl radical formation (Platenik *et al*., 2001). The QUIN-Fe^{2+} complex was shown to be responsible for *in vitro* DNA chain breakage and lipid peroxidation mediated by hydroxyl radicals (Goda *et al*., 1996). The ratio of Fe^{2+}: Fe^{3+} ions is critical for the initial rate and extent of lipid peroxidation, the most potent ratio being 1:1. QUIN reduces this ratio by complexing the Fe^{2+} ions, and ascorbate, or an equivalent reducing agent, increases the ratio by reducing Fe^{3+} to Fe^{2+} ions (Stipek *et al*., 1997).

Iron is normally present in the body in a highly bound state, often bound to proteins such as transferrin and ferritin. However, there is a small pool of non-protein bound iron in the body, and free iron has been detected in the CSF (2.2μM) but not in serum or plasma (Halliwell and Gutteridge, 1984). This level of free iron is sufficient to react with QUIN to stimulate lipid peroxidation (Stipek *et al*., 1997). However, in conditions of oxidative stress, superoxide or other reducing agents in the body can release Fe^{2+} ions from bound Fe^{3+} ions (Halliwell and Gutteridge, 1984). QUIN concentrations of approximately 100μM (Stipek *et al*., 1997; Leipnitz *et al*., 2005) were required to interact with Fe^{2+} ions and stimulate lipid peroxidation, which are much higher than the levels present in the human brain (<40nM in CSF), with CSF levels increasing to a mean of 1.3μM for patients with the AIDS-dementia complex (Heyes *et al.*, 1991a). However, with the activation of IDO activity, QUIN concentrations could potentially increase to levels which would stimulate lipid peroxidation in discrete brain areas (Stipek *et al*., 1997).

It is still unclear whether QUIN-induced lipid peroxidation involving its chelation with iron is independent from QUIN activation of NMDA receptors. Leipnitz *et al*. (2005) prepared rat cerebral cortex homogenates free of nuclei and cell debris, therefore not containing NMDA receptors, and still demonstrated that QUIN addition induced lipid peroxidation and a reduction in antioxidants. The antioxidant, melatonin, was able to prevent QUIN but not NMDA neuronal damage in the rat hippocampus, showing that the QUIN toxicity was, at least in part, independent of NMDA receptor activation (Behan *et al*., 1999). It is therefore unlikely that the significant induction of oxidative stress by QUIN was due to NMDA receptor activation causing free radical synthesis. However, NMDA receptor inhibitors were shown to block QUIN-induced lipid peroxidation, with trace amounts of Fe^{2+} ions present. QUIN-induced lipid peroxidation was prevented by KYNA *in vitro* (Rios and Santamaria, 1991) and by the non-competitive NMDA receptor antagonist, MK-801, *in vivo* (Santamaria and Rios, 1993). However, with added Fe^{2+} ions, the QUIN-Fe^{2+} complex inhibited the binding of radiolabelled MK-801 (St'asny *et al*., 1999). An alternative approach was used by Behan and Stone (2002) where the neurotoxicity of QUIN was shown to be increased when a source of free radicals were injected with QUIN into the rat hippocampus. Both xanthine/xanthine oxidase mixture and the nitric oxide donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), as sources of free radicals, increased the neuronal damage produced by QUIN. The NMDA receptor antagonist, 5,7-dichlorokynurenic acid, prevented the damage by QUIN alone but not the damage produced by QUIN and xanthine/xanthine oxidase, showing that the damage was not simply caused by free radical enhancement of NMDA receptor activation. It may be that

hydroxyl radical formation has to be instigated by hydroxyl radical attack of QUIN. Under the experimental conditions in the healthy rat as described by Behan and Stone (2002), these initial hydroxyl radicals may result from NMDA receptor activation by QUIN, but in a disease state, the initial hydroxyl radicals could be from a range of sources. In neurological disorders, the damage produced by QUIN is partly dependent on the inflammatory response, where activated microglia and macrophages infiltrate the brain and produce reactive oxygen species (Behan *et al*., 1999). This extra contribution of reactive oxygen species could act with QUIN to produce neuronal damage. Free radicals themselves enhance the release of glutamate from the synapse and this effect could lead to maintaining NMDA receptor activation. It can be concluded that the high potency of QUIN toxicity to neurones is the result of synergism between the activation of NMDA receptors and the formation of free radicals. The combined mechanisms may be required for neuronal death (Behan *et al*., 1999).

In contrast to the effect of Fe^{2+} ions, copper ions (Cu^{2+} ions) were shown to protect against the neurotoxicity of QUIN in the rat striatum (Santamaria *et al*., 2003). Intraperitoneal injection of copper sulphate 30 minutes before an intrastriatal injection of QUIN reduced QUIN excitotoxicity measured both as circling behaviour of the rats and depletion in striatal GABA content, suggesting that Cu^{2+} ions were inhibiting QUIN's activation of NMDA receptors. It has been suggested that Cu^{2+} ions may act on an extracellular binding site of the NMDA receptor, probably a redox-modulatory site. In addition, Cu^{2+} ions blocked the striatal lipid peroxidation and reverted the decreased levels of CuZn-SOD back to normal in these QUIN-treated rats. Therefore, Cu^{2+} ions also had antioxidant properties. Free reduced copper ions (Cu^{+}) are in short supply in the CNS and this may be the reason why copper does not participate in the Fenton reaction in a similar way to Fe^{2+} ions. Indeed copper is almost exclusively bound to proteins (Halliwell and Gutteridge, 1984). Cu^{2+} ions were shown to increase the ferroxidase activity of ceruloplasmin (a protein containing bound copper) in the CSF of rats after QUIN injection (Santamaria *et al*., 2003), suggesting an interaction between copper and iron ions, which may affect the oxidative activity in the brain. This interaction has yet to be fully investigated. It was interesting that the higher doses of Cu^{2+} ions (7.5 and 10.0mg/kg) used in this study were toxic to the rats (Santamaria *et al*., 2003).

QUIN excitotoxicity can be modulated by other endogenous kynurenines. With focal injections into the rat nucleus basalis magnocellularis, Jhamandas *et al*. (1990) demonstrated that although KYNA was the most effective against QUIN-induced cholinergic damage, giving complete protection at a ratio of 1:1, picolinic acid could also

completely prevent such damage but at a much higher ratio of 1:4 (QUIN: picolinic acid). The neuroprotective effect of picolinic acid shown in this study is inconsistent with the results of Robinson *et al*. (1985), where picolinic acid was poor as an antagonist at synaptically evoked responses in rat hippocampal slices, which could be blocked by KYNA. Indeed, picolinic acid did not effectively block QUIN-induced damage to striatal cholinergic neurones (Jhamandas *et al*., 1990). The neuroprotective effect of picolinic acid does not seem to occur as an interaction with NMDA receptors. ANA and quinaldic acid were shown to give partial protection against QUIN-induced neuronal damage. In certain neurodegenerative disorders, the balance of endogenous excitotoxins and antagonists (including KYNA, picolinic acid and ANA) may influence neuronal survival. However, another kynurenine metabolite, 3HKYN, enhanced QUIN toxicity (Guidetti and Schwarcz, 1999).

1.1.14 Synergy between 3-Hydroxykynurenine and Quinolinic Acid

In rats, intrastriatal co-injections of 5nmoles 3HKYN and 15nmoles QUIN, doses which caused no degeneration on their own, together showed substantial neuronal loss 4 days after injection, demonstrated behaviourally (apomorphine-induced rotations) and histologically (lesion volume) (Guidetti and Schwarcz, 1999). This suggests that even at low concentrations of QUIN, there is a synergistic effect with 3HKYN, which can cause neurodegeneration. The lesions were excitotoxic in nature as an NMDA inhibitor (CGP 40116) prevented lesion formation and were qualitatively indistinguishable from those produced by a higher dosage of QUIN alone. 3HKYN can be converted into QUIN in the brain, but it was shown that this was not occurring as there was no change in QUIN or 3HKYN levels over time. A free radical scavenger, *N*-tert-butyl-α-(2-sulphophenyl)-nitrone (S-PBN) also inhibited lesion formation, demonstrating the necessity of free radicals for neuronal loss. 3HKYN did not enhance the toxicity of NMDA in the rat striatum, when QUIN was substituted by NMDA, suggesting that there may be a joint effect of 3HKYN and QUIN in generating sufficient free radicals to cause the neurodegeneration. Other free radical scavengers, indolepropionic acid and PNU 101033, were also able to reduce the increase in lesion volume caused by the addition of 3HKYN to a QUIN intrastriatal injection in rats (Guidetti and Schwarcz, 2003). These free radical scavengers failed to block the neurotoxicity of QUIN alone. However, the NMDA receptor antagonist, MK-801, completely prevented neuronal loss induced by QUIN and 3HKYN together. These results showed that 3HKYN potentiates striatal QUIN toxicity

and these compounds may act together to contribute to neuronal damage in neurological disorders. This enhanced toxicity appears to be due to 3HKYN, and perhaps QUIN also, generating free radicals, but requires NMDA receptor activation.

1.1.15 Mechanisms of Cell Death initiated by 3-Hydroxykynurenine and Quinolinic Acid

There are various contrasting opinions on the neurotoxic potency of 3HKYN and QUIN. Neurotoxicity of 3HKYN and QUIN were similar *in vitro* (Chiarugi *et al*., 2001). However, the lesion size of an intrastriatal injection in rats of 50nmoles 3HKYN was similar to that induced by 200nmoles QUIN (Nakagami *et al*., 1996).

The mechanism for neuronal cell death caused by 3HKYN or QUIN has been investigated in primary mixed cortical cell cultures (Chiarugi *et al*., 2001). It was suggested that QUIN neurotoxicity is caused by necrosis, whereas with 3HKYN toxicity, neurones die by apoptosis. This confirmed the results of Okuda *et al*. (1998), where 3HKYN was shown to cause apoptosis. NMDA receptor antagonists, neuronal NOS (nNOS) inhibitors and poly (adenosine diphosphate (ADP) -ribose) polymerase (PARP) inhibitors all can prevent QUIN-induced toxicity, but were not able to reduce 3HKYN toxicity (Chiarugi *et al*., 2001). This suggests that QUIN neurotoxicity involves NMDA receptor-mediated activation of nNOS, formation of DNA nicks, hyperactivation of PARP resulting in energy failure and finally necrotic-type cell death. Five different free radical scavengers reduced 3HKYN toxicity, but only three reduced QUIN toxicity, showing that free radicals are involved in both mechanisms of cell death. The enzyme, caspase-3, has a key role in the execution phase of apoptosis and an increase in its activity is a marker for an ongoing apoptotic process. With 3HKYN, cells showed an increase in caspase-3 at 24h, remaining elevated for up to 72h exposure, whereas with QUIN, there was no increase in caspase-3. The opening of the mitochondrial permeability transition pore may trigger the execution of the apoptotic process. Cyclosporin can reduce the opening of this channel. Cyclosporin was shown to reduce the toxicity of 3HKYN, but not of QUIN, again suggesting that 3HKYN neurotoxicity, but not that of QUIN, involves apoptosis. ATP availability was also examined after various times of exposure to the neurotoxins. QUIN depleted the energetic stores more rapidly than 3HKYN, suggesting that QUIN-induced cell death was more likely to be by necrosis, and not by apoptosis, as ATP is required in some steps of apoptotic cell death (Chiarugi *et al*., 2001).

1.1.16 Location of Kynurenine Pathway Enzymes

The enzymes of the kynurenine pathway are present in the brain, but have much lower activity than in the peripheral organs. The enzymes involved in the metabolism of tryptophan to produce QUIN have been confirmed to be identical in the brain and the periphery, with the same high substrate specificity and high substrate affinity. Although some enzymes show several-fold differences between brain regions, and activities are variable depending on species and age, no clear specific functions relating to these differences have emerged to date (Schwarcz and Pellicciari, 2002).

The KAT enzymes are primarily located in astrocytes (Guillemin *et al*., 2001), although KAT has also been found in medium and large neurones in the striatum (Roberts *et al*., 1992). Recently, a new KAT II antibody has been used for immunocytochemistry, which has confirmed the localisation of KAT II in astrocytes, with no KAT II detected in neurones or microglia (Guidetti *et al*., 2007b). KAT II-positive astrocytes were associated with select neurone populations, supporting a role for KYNA in mediating neuronal activity. Intense staining for KAT II was frequently observed around brain capillaries, which may explain the rapid formation of KYNA in the brain when kynurenine levels are elevated in the blood.

3HAO is located primarily in astrocytes in all regions of the rat brain, although some neurones have been shown to contain 3HAO (Okuno *et al*., 1987; Kohler *et al*., 1988). QPRT exists primarily in glial cells but again there is some present in neurones in the human striatum (Du *et al*., 1991). There are substantially fewer cells that contain QPRT than contain 3HAO (Kohler *et al*., 1988), suggesting that QUIN can be produced more quickly than it can be metabolised.

It is now generally established that astrocytes and microglia, rather than neurones, contain the enzymes required for the biosynthesis of kynurenines (Schwarcz and Pellicciari, 2002).

Human astrocytes in cultures stimulated with a range of cytokines involved in brain inflammation to induce IDO, lack KMO and hence cannot convert kynurenine into 3HKYN, and therefore produce large amounts of kynurenine and KYNA (Guillemin *et al*., 2001). These human astrocytes express most of the other kynurenine pathway enzymes, although there is some discrepancy between studies as to whether kynureninase is expressed. With the lack of KMO, it may be expected that some kynurenine would be converted to ANA by kynureninase, but this was not observed by Guillemin *et al*. (2001).

Astrocytes demonstrated high QPRT activity, which completely degraded the small amounts of QUIN synthesised and also much of the additional QUIN (1μM) when it was supplied to the cell culture. Astrocytes can therefore metabolise most of the QUIN formed, and therefore QUIN is not available to cause excitotoxicity. The addition of kynurenine to macrophages, at the level produced by the cytokine-stimulated human astrocyte cultures, led to a significant production of QUIN. As astrocytes are not isolated from infiltrating macrophages following an immune stimulus, the production of kynurenine in astrocytes can contribute to excessive QUIN production in macrophages.

Following an immune or inflammatory response, infiltrating macrophages and resident microglial cells are both able to convert tryptophan into QUIN. High concentrations of QUIN were produced in human blood macrophage cultures and human foetal microglia cultures after stimulation with IFN-γ. In contrast, cultures of human foetal astrocytes and neurones, stimulated with IFN-γ produced negligible amounts of QUIN (Heyes *et al*, 1996). Guillemin *et al*. (2003) demonstrated the expression of kynurenine enzymes in human microglia and macrophages. The enzymes KAT I, KAT II, kynureninase, KMO, 3HAO and QPRT were all expressed in unstimulated macrophages and microglia, although there was no expression of IDO. The amount of kynureninase and KMO mRNA were 10-fold and 5-fold higher, respectively, in macrophages compared with microglia. After IFN-γ stimulation, IDO was found in macrophages and microglia, with macrophages expressing three times more IDO than microglia. The amount of IDO transcripts was far higher than those of the other kynurenine pathway enzymes examined. In microglia, stimulation with IFN-γ did not increase the expression of any of the other kynurenine pathway enzymes, however, in macrophages, there was a small increase (2-fold) in expression of KAT I, KAT II and kynureninase. Substantially less QUIN (20-fold) was produced from microglia than from macrophages after IFN-γ stimulation, which can be attributed to the lower expression of IDO, kynureninase and KMO in microglia (Guillemin *et al*., 2003).

1.1.17 Transport between the Brain and the Periphery

The microvasculature of the CNS supplies blood to brain structures, with a barrier between its microvessels and the brain tissue. This blood-brain barrier is composed of endothelial cells, functioning by their cohesive and resistance properties which require astrocytic co-operation and vascular matrix adhesion (del Zoppo *et al*., 2006).

Fukui *et al*. (1991) determined the rates of cerebral uptake and mechanisms of blood-brain barrier transport for the main kynurenines in rats. Tryptophan and kynurenine can readily cross the blood-brain barrier, being transported by the large neutral amino acid carrier (L-system). Kynurenine, an α-amino acid, has a free carboxyl group and an unsubstituted α-amino group that are required for L-system activity. Tryptophan and leucine can inhibit the blood-brain transport of kynurenine by at least 90%, indicating that the α-amino acids act competitively for this carrier. 3HKYN appeared to be transported by the same carrier, but the affinity for 3HKYN was lower than that of kynurenine due to the presence of the additional hydroxyl group. For the other metabolites, uptake appeared to be mediated by passive diffusion, which occurred at a significant rate for ANA, but at a far lower rate for 3HANA, KYNA and QUIN. It was concluded that kynurenine crosses the blood-brain barrier easily, 3HKYN and ANA may also cross and contribute to cerebral levels, but 3HANA, KYNA and QUIN cross the blood-brain barrier poorly and are not expected to contribute to brain pools. However, in healthy gerbils with labelled QUIN infused subcutaneously, Heyes and Morrison (1997) showed that 38-49% of QUIN in the brain was derived from the blood. It is therefore unclear whether blood QUIN does contribute to brain QUIN levels.

Fukui *et al*. (1991) used normal healthy rats to examine the transport of kynurenines across the blood-brain barrier, but when considering neurological disorders, such as ischaemia and TBI, there is degradation of the blood-brain barrier (Skinner *et al*., 2006; Habgood *et al*., 2007). This will enable kynurenines to pass more freely from the blood to the brain.

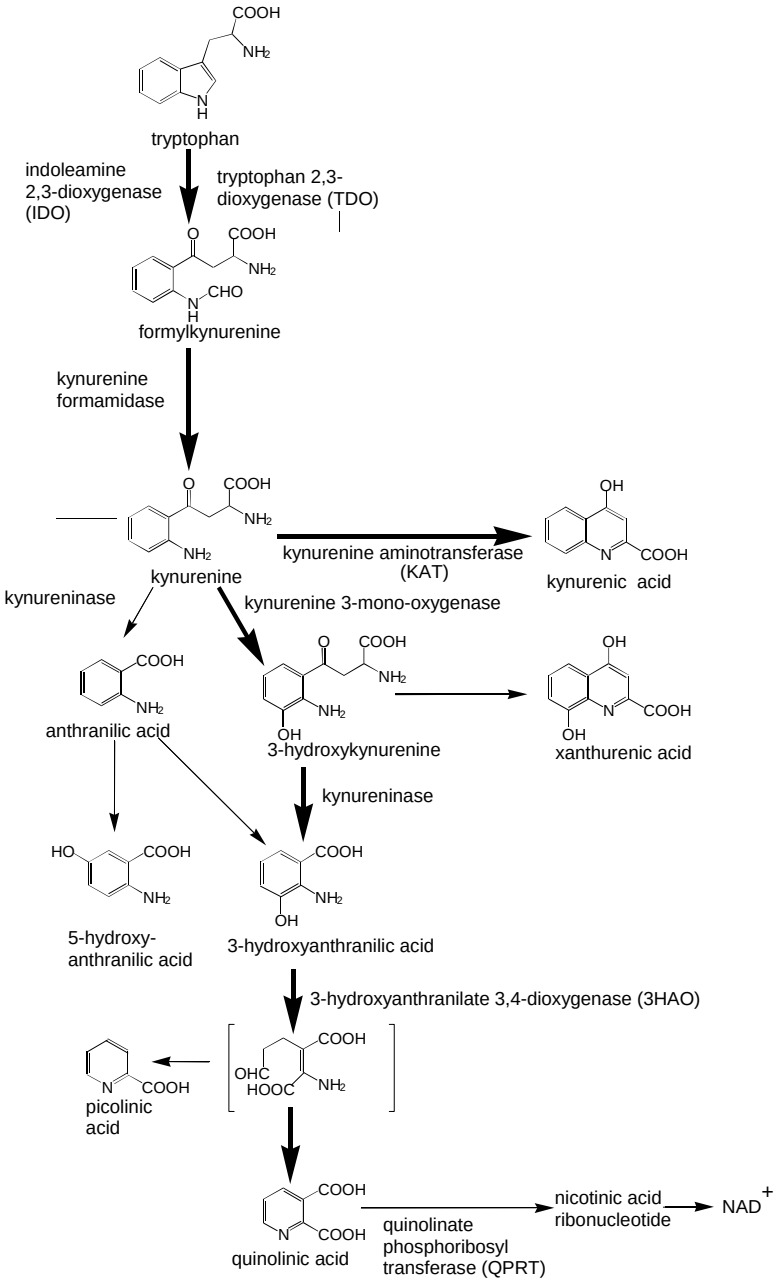


Figure 1-1 A summary of the key components of the kynurenine pathway.

(Courtesy of T.W. Stone)

1.2 Neurological Disorders

1.2.1 Neurodegeneration

The kynurenine pathway is activated in a variety of inflammatory diseases affecting the brain including meningitis, septicaemia and neurovirological disorders such as the AIDS-dementia complex (Heyes *et al*., 1992a) and implicated in neurological disorders, such as HD, epilepsy, TBI and ischaemic stroke (Stone, 2001).

Neurodegenerative disorders have slow progression and are frequently hereditary. These diseases show a selective loss of a particular subset of neurones for unknown reasons. Although the initial trigger of neuronal death is not clear, the consequent events can proceed gradually or episodically. Each neurone has its own optimal biochemical conditions, such as intracellular pH, water content, concentrations of oxygen, glucose, ATP and calcium ions. An imbalance in these conditions can cause neuronal damage or death. Apoptosis has been implicated as a possible mechanism for neuronal death in neurodegenerative disorders. However, there is no direct and substantial evidence of apoptosis in human brains, with apoptosis in developing nervous systems being much quicker than the extremely slow neuronal loss in neurodegenerative disorders (Kanazawa, 2001).

During the normal development of the nervous system, over half of the neurones of the central and peripheral nervous systems die. Neurones are lost by apoptosis, a process of programmed cell death. These cells have differentiated and successfully extended their axons to their target cells. The target tissue limits the number of axons attaching to it, and hence rejects some neurones and causes apoptosis of the whole neurone. Surviving neurones must make a connection with the target cells, with the target tissue able to transport substances vital for neuronal survival. Substances transported from the target tissue to the neuronal cell body are called neurotrophic factors and are produced by the target cells in limited amounts. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are two of many regulatory neurotrophic factors, with each factor favouring the survival of a different group of neurones. BDNF can rescue foetal motor neurones from naturally occurring cell death and from induced cell death by the removal of the target tissue (Kanazawa, 2001).

In response to neuronal injury, glial cells alter their morphology, size and number and also increase the production and release of cytokines and growth factors (Aschner *et al*., 2002;

Kim *et al*., 2003). This stimulation induces IDO activity and the rapid up-regulation of the 3HKYN/QUIN branch of the kynurenine pathway within reactive microglial cells. KYNA formation, which takes place mainly in astrocytes (Kiss *et al*., 2003), is far less affected by exposure to cytokines with increases in CSF QUIN: KYNA ratios occurring after immune activation (Heyes *et al*., 1992a).

A major consequence of reactive oxygen species activity in living tissue is the oxidation of cell membrane lipids. Brain neuronal cells are at particular risk from damage caused by free radicals, since the brain has an extremely high rate of oxygen consumption that can lead to increased superoxide formation and neuronal membranes have high contents of polyunsaturated fatty acids that are susceptible to lipid peroxidation (Okuda *et al*., 1996). In addition, the brain has modest antioxidant defences and there is high Ca^{2+} ion transport across neuronal membranes (Leipnitz *et al*., 2005). Under physiological conditions, there is a well-balanced equilibrium between free radical generation and various enzymatic and non-enzymatic antioxidant defense systems. Oxidative stress occurs when there is an imbalance towards free radical accumulation and has been implicated in several neurodegenerative disorders (Coyle and Puttfarcken, 1993). Cumulative damage caused by reactive oxygen species may explain the delayed onset and progressive characteristics of cell death in such diseases. Oxidative stress can be triggered either by impairment of antioxidant defense mechanisms or by increased generation of reactive oxygen species. 3HKYN has been proposed as a factor that contributes to reactive oxygen species generation in the brain (Okuda *et al*., 1998).

Mitochondrial dysfunction and excitotoxicity appear to be closely related in neurodegeneration and it has been hypothesised that bioenergetic defects could lead to neuronal death via secondary excitotoxicity (Browne *et al*., 1999). Impaired mitochondrial energy metabolism, reducing ATP production, can cause neurones to be more vulnerable to endogenous levels of excitatory amino acids and result in partial cell depolarisation. As more calcium ions enter the neurones, this may trigger further free radical production, exacerbating the damage to cellular components. This hypothesis has been supported with work demonstrating that normal levels of glutamate become toxic in the presence of, for example, oxidative phosphorylation inhibitors (Novelli *et al*., 1988), and excitatory amino acid antagonists such as MK-801 can reduce cerebral lesions induced by mitochondrial toxins (Beal *et al*., 1991). It remains unresolved whether bioenergetic dysfunction or oxidative damage are causative factors or occur secondarily to neuronal loss in HD.

Neopterin is used as a marker of disease as it indicates an activated cellular immune system in humans (Hoffmann and Schobersberger, 2004). Following bacterial infections, activated TH1-type lymphocytes release IFN-γ, which activates monocytes and macrophages to produce and release neopterin. Neopterin itself, or together with cytokines, triggers pro-inflammatory events, including the expression of iNOS and tumour necrosis factor- α (TNF-α) synthesis. One outcome of this is the excessive production of nitric oxide leading to oxidative stress. Neopterin has also been shown to induce apoptosis, where it may (in vascular smooth muscle cells) or may not (in inflammatory diseases of the lung) be related to nitric oxide production. In the alveolar epithelium, neopterin has also been shown to have a role in upregulating the synthesis of intracellular adhesion molecule-1, which mediates the migration and activation of leukocytes to the site of infection. Chronically increased activities of the cellular immune system are often associated with anaemia. Neopterin may also be involved in the onset of anaemia, without a link with oxidative stress. Neopterin can cause an increase in intracellular Ca^{2+} ions, but this appears to be cell-type specific. The precise role and importance of neopterin in infection and inflammation remains unclear, but it appears to be stimulated by IFN-γ and acts as a mediator of host-defence reactions, often associated with nitric oxide (Hoffmann and Schobersberger, 2004).

1.2.2 Huntington's Disease

1.2.2.1 General

HD is an inherited neurodegenerative disorder that affects four to ten per 100,000 individuals of Caucasian origin (Reddy *et al*., 1999). The disease is characterised by neuronal loss in the striatum and cortex, although there are minor volume reductions in almost all brain structures, observed using magnetic resonance imaging (MRI) coronal scans, which occur even for individuals with early to mid-stages of HD (Rosas *et al*., 2003). The symptoms are progressive chorea (involuntary body movements of the arms, legs, face, mouth or torso), progressive cognitive impairment leading to dementia and emotional disturbances. The onset of the disease usually occurs between the ages of 35 and 42, with symptoms becoming more severe until death, generally about 17 years after the first manifestation of symptoms (Martin and Gusella, 1986).

HD is an autosomal dominant disorder, and hence a sufferer of the disease has a 50% risk of passing the condition on to their offspring, and both males and females can equally contract the condition. The gene responsible for HD has been identified (The Huntington's

Disease Collaborative Research Group, 1993) and has an expansion of CAG repeats that codes for the amino acid glutamine. In the general population, the number of CAG repeats ranges from 6 to 35 whereas HD sufferers have between approximately 40 and 121. This gene encodes the 349kDa protein, named huntingtin, where in HD, there are usually greater than 35 glutamine repeats at its amino terminus. The length of the CAG repeats is related to the age of onset of the disorder (Reddy *et al*., 1999). The earlier the age of onset of the disease, the faster the disease progresses and the greater the severity of the symptoms. Early-onset HD is associated with paternal transmission whereas late-onset is most frequently linked to maternal transmission (Martin and Gusella, 1986). Mothers may be able to pass on protective factors to the foetus via the placenta during pregnancy, thereby protecting the basal ganglia and postponing neurodegenerative changes.

Post-mortem HD brains have been graded from grade 0 to grade 4, according to the neuropathological severity in the striatum (Vonsattel *et al*., 1985). Brains which showed no gross or microscopical abnormalities in the striatum, but there had been substantial clinical evidence for the diagnosis of HD, were graded 0. Grade 1 HD brains showed no macroscopic alterations in the striatum but microscopic examination showed 50% neuronal loss in the caudate nucleus. At grade 2, atrophy at the head of the caudate nucleus was observed macroscopically. Neuronal loss in the striatum increased with increasing grades. For grade 3 brains, the caudate nucleus was markedly shrunken, with a moderate decrease in the size of the putamen and globus pallidus, observed macroscopically. By grade 4, brains had further degraded with the loss of 95% of the neurones in the caudate nucleus. The grade correlated closely with the rating of physical disability at the time of death (Vonsattel *et al*., 1985).

1.2.2.2 The Role of the Huntingtin Protein

The mechanisms by which huntingtin causes neuronal damage have been investigated but remain unresolved. The reason for the preferential susceptibility of striatal neurones is also unknown. Neurodegeneration may originate in one vulnerable brain area and nerve cells may become damaged independently from each other or through pathogenic cell-cell interactions. Much work, which is still ongoing, has studied the roles of huntingtin aggregation, intranuclear inclusions and the proteolytic generation of toxic huntingtin fragments. Intranuclear aggregates in small striatal neurones have been found in a transgenic mouse model of HD (Kanazawa, 2001). Aggregates are also found in the post-mortem striatum and cortex in HD. However there does not seem to be a correlation between the formation of inclusion bodies and neuronal cell death in cultured neurones

expressing abnormal huntingtin (Saudou *et al.*, 1998). Proposed roles for aggregates of huntingtin, found in post-mortem brains, have ranged from being critical for disease pathogenesis, to being a benign phenomenon, and even being neuroprotective (Bates 2003). Yang *et al*. (2002) demonstrated that preformed aggregates are highly toxic when directed to the cell nucleus. Consistent with this, pharmacological intervention to inhibit aggregate formation has shown beneficial effects in a mouse model of HD (Sanchez *et al*., 2003). Huntingtin aggregates may not directly cause neuronal damage, however, they do appear to have a major role in the pathogenesis of HD.

In a transgenic HD mouse model, where the mice demonstrate neuronal inclusions, characteristic neuropathology and progressive motor dysfunction, it has been shown that it is possible to turn off the expression of the transgene with oral administration of tetracycline analogues. This leads to a disappearance of inclusions and an improvement in the motor function of these mice (Yamamoto *et al*., 2000). Although impaired surviving neurones are generally thought to die eventually, there is the possibility that they could revert to their normal healthy state (Kanazawa, 2001). A continuous influx of the mutant protein is required to maintain inclusions and symptoms, suggesting that HD may be reversible (Yamamoto *et al*., 2000).

Apoptosis has been implicated as the mechanism of cell death in HD (Portera-Cailliau *et al*., 1995) but there is no conclusive evidence of apoptosis in the HD human brain (Kanazawa, 2001). Blocking apoptosis may help to halt the onset and symptoms of HD. The activation of caspase-1, a protease involved in triggering apoptosis in cells, has been shown to have the ability to cleave huntingtin. Transgenic mice with the huntingtin mutation (R6/2 mice) were cross-bred with transgenic mice lacking caspase-1 activity and the cross-bred mice had a delayed disease onset and survived longer than mice with the huntingtin mutation only (Ona *et al*., 1999). A caspase inhibitor was also given to the R6/2 mice and this slowed the disease progression and delayed mortality. This work suggested that the mutant huntingtin protein causes apoptosis by inducing a toxic state inside the cell which activates caspase-1. Caspase-1 could cleave the mutant huntingtin, generating toxic huntingtin fragments and set up a feedback loop that eventually leads to cell dysfunction and death (Ona *et al*., 1999). This idea of the toxicity building up over time could start to explain why the symptoms of HD do not manifest themselves until mid-life, although the gene is present from birth.

1.2.2.3 Neuronal Damage and related Animal Models of Huntington's Disease

HD neuropathology is characterised by bilateral striatal atrophy with marked neuronal loss and astrogliosis within the caudate and putamen (Browne *et al*., 1999). The striatal cell type most susceptible to degeneration is the medium spiny projection neurone. 80% of striatal neurones are spiny neurones and these are the principal input and output neurones of the striatum. The other major class of striatal neurones are the aspiny interneurones. NADPH-diaphorase, neuropeptide Y, somatostatin and NOS typically colocalise in medium aspiny neurones. The large aspiny neurones contain acetylcholine.

HD affects several neurotransmitter systems. One of these is a reduction in the inhibitory transmitter, GABA, observed within the striatal spiny neurones (Browne *et al*., 1999). This reduces GABA-mediated inhibition of the substantia nigra, and increases dopamine. There is then an imbalance in the dopamine-acetylcholine relationship in the striatum, which is thought to cause the characteristic uncontrollable choreic movements in HD (Martin and Gusella, 1986). At present, one successful treatment of HD is with dopamine antagonists which appear to relieve the choreic symptoms.

The original animal model for HD proposed by Coyle and Schwarcz (1976) and McGeer and McGeer (1976) used intrastriatal injections of kainic acid, which produced local specific degeneration of GABAergic neurones, with preservation of dopaminergic neurones. The rats also showed similar histological and behavioural changes to those observed in HD.

In the striatum in HD, there is an increase in somatostatin and neuropeptide Y, which results in the preservation of a subclass of striatal aspiny neurones. Beal *et al.* (1986) demonstrated that lesions produced by QUIN resemble those of HD, with a depletion of GABA and substance P and selective sparing of somatostatin/ neuropeptide Y neurones. This could be attributed to NMDA receptor-induced excitotoxicity, as NMDA injections also preserved these neurones. Lesions produced by kainic acid, ibotenic acid, quisqualic acid or AMPA were unlike those produced by QUIN, as they affected all cell types, without sparing somatostatin/ neuropeptide Y neurones (Beal *et al*., 1989). Following QUIN excitotoxicity in the rat, cholinergic neurones in the striatum were also relatively spared (Davies and Roberts, 1988), although the same effect was also observed with other excitotoxins acting at NMDA, AMPA and kainic acid receptors (Beal *et al*., 1989). In HD, cholinergic neurones were also relatively preserved (Ferrante *et al.*, 1987). These reports

suggested a more accurate animal model for HD is by injection of QUIN into the striatum rather than kainic acid. Such a QUIN model also seems a more realistic model for HD as QUIN is an endogenous compound in the mammalian brain whereas kainic acid is not present.

The relatively resistant medium aspiny neurones also contain NOS. NOS-containing neurones in the striatum are known to be resistant to acute excitotoxic insults and nitric oxide toxicity which may be due to their antioxidant properties (Browne *et al*., 1999). Cultured nitric oxide-resistant neurones were found to contain elevated levels of the mitochondrial superoxide radical scavenging enzyme, Mn-SOD, which was critical for the resistance of these neurones to nitric oxide- and NMDA- toxicity (Gonzalez-Zulueta *et al*., 1998).

1.2.2.4 Quinolinic Acid Hypothesis for Huntington's Disease

The first hypothesis for the mechanism of neuronal loss in HD involving activation of the kynurenine pathway suggests an excessive stimulation of NMDA receptors by QUIN and then a subsequent increase of intracellular calcium (Beal *et al*., 1986). This hypothesis was supported by Young *et al.* (1988) who showed that NMDA receptor binding was reduced by 93% in the putamen of HD post-mortem brains compared to binding in control brains, a significantly greater decrease than observed for other receptors in the putamen. There was no effect on NMDA receptor binding in the cerebral cortex. This work demonstrates that cells with high densities of NMDA receptors are preferentially lost in HD striatum. In support of this finding, a significant loss in binding to the glycine site of the NMDA receptor complex in HD brains was observed, particularly in the caudate nucleus but also in the frontal cortex (Reynolds *et al.,* 1994).

The hypothesis suggests that brain tissue in HD will contain an elevated level of QUIN. Furthermore, the activity of 3HAO, the enzyme which synthesises QUIN from its kynurenine pathway precursor, 3HANA, has been shown to be increased in the HD brains (Schwarcz *et al*., 1988a). However, no increase in QUIN in the CSF was found, with concentrations unexpectedly being slightly but not significantly lower than in control CSF (Heyes *et al.,* 1991b). Similarly, levels of QUIN appeared to be slightly lower, rather than higher, in HD brain tissue. These reduced levels of QUIN were only significant in 3 out of the 6 areas of the cerebral cortex examined, with levels in the putamen, dentate nucleus of the cerebellum and other cortical regions slightly reduced but not significantly different from controls. These results are in agreement with previous studies, where similar levels

of QUIN in CSF in HD patients were shown compared with controls (Schwarcz *et al*., 1988b), and no increased levels of QUIN were found in HD brain (Reynolds *et al*., 1988).

1.2.2.5 Kynurenic Acid Hypothesis for Huntington's Disease

An alternative hypothesis for excessive stimulation of NMDA receptors may be a reduction in KYNA instead of an increase in QUIN. KYNA acts as an antagonist at NMDA receptors, and neurotoxicity could occur when there is a decrease in brain KYNA levels. However, Connick *et al*. (1989) showed increased KYNA in the motor cortex of post-mortem HD brains, with no differences in KYNA levels in the prefrontal cortex, the caudate nucleus or globus pallidus compared to controls. It was suggested that the increase in KYNA may not be a primary effect, but a compensatory response to an increase in excitatory activity.

Other groups however demonstrated a decrease in KYNA or a decrease in the formation of KYNA from its bioprecursor, kynurenine. In post-mortem human brains with HD, there was a significant increase in the ratio of kynurenine: KYNA and there was also a reduced concentration of KYNA in the CSF of HD patients (Beal *et al.,* 1990). They suggested that if KYNA regulates by blocking NMDA receptor activation, a chronic deficiency could potentially lead to a slowly developing NMDA excitotoxic neurodegenerative process. Further work showed a decrease in the levels of KYNA in HD cerebral cortex (Beal *et al.,* 1992). This effect may be important over a period of time but the reported concentrations of KYNA required to block NMDA receptor activation are about 100-fold greater than those found in the human brain (Jahr and Jessel, 1985).

Jauch *et al.* (1995) measured KYNA levels in 12 brain regions from late-stage HD brains and the activities of the two KYNA synthesising enzymes, KAT I and KAT II. The only region of the brain that showed a significant reduction in the level of KYNA was the caudate nucleus. The putamen and globus pallidus also had reduced KYNA levels but these were not significant. However, the only brain region which showed significantly reduced KAT I and KAT II activities was the putamen, which also had approximately 3-fold higher Km for both enzymes. These results suggest a selective impairment in KYNA biosynthesis, which may be due to the loss of endogenous KAT activators. However, Pearson *et al*. (1995) found no change in KAT activity in HD brain in the caudate or frontal cortex.

1.2.2.6 3-Hydroxykynurenine Hypothesis for Huntington's Disease

Another neurotoxic metabolite of the kynurenine pathway has also been implicated in HD. HD post-mortem brain showed 2-3-fold increases of 3HKYN in cortical and striatal regions (Reynolds and Pearson, 1989; Pearson and Reynolds, 1992). However, Beal *et al*. (1990) did not observe any changes in 3HKYN levels in HD putamen. Acute intracerebroventricular injection of 3HKYN is known to have a toxic effect in animals, causing seizures (Lapin, 1981). Elevated levels of 3HKYN have also been observed in other diseases where neurological function is impaired: hepatic encephalopathy (Pearson and Reynolds, 1991) and infantile spasms (Yamamoto, 1991). There was some debate about the relevance of the toxicity of long-term low-dose exposure to 3HKYN in HD and the unknown factors which may modulate its *in vivo* toxicity (Guilarte and Eastman, 1993). The neurotoxic effects of 3HKYN have only been demonstrated using high micromolar concentrations in a neuronal cell line. However, 3HKYN causes the death of neuronally-derived cells in culture at concentrations lower than other excitotoxins, including QUIN and glutamate (Eastman and Guilarte, 1989). Together with the presence of endogenous ascorbic acid and iron, the toxicity of 3HKYN may be enhanced *in vivo*. Guilarte and Eastman (1993) suggested that further work is required to demonstrate whether 3HKYN is an endogenous neurotoxin, relevant in HD.

The suggestion of high levels of 3HKYN in the brain acting as a neurotoxin involved in HD leads to consideration of where the 3HKYN is produced. The enzymes responsible for the synthesis of 3HKYN are at much lower levels in brain than in peripheral tissues, such as the liver and kidney (Erickson *et al*., 1992). As 3HKYN can cross the blood-brain barrier, 3HKYN from the blood may well contribute to the cerebral pool of 3HKYN. Interestingly, the striatum and cortex have the highest capacity for 3HKYN uptake (Eastman *et al*., 1992) and are the same regions where elevated 3HKYN is found in the HD brain (Pearson and Reynolds, 1992). Similarly, elevations of 3HKYN in the blood could possibly act as a peripheral marker of HD.

1.2.2.7 Alterations in the Different Branches of the Kynurenine Pathway as a Hypothesis for Huntington's Disease

Guidetti *et al*. (2000) proposed a refined hypothesis for the involvement of kynurenines in HD: "An increased flux through the 3HKYN/ QUIN branch compared to the KYNA branch of the kynurenine pathway may play a causative role in HD pathology." Increased levels of 3HKYN and KYNA were demonstrated in the striatum of post-mortem brains of

grade 1 HD patients who had died in the early stage of neurodegeneration. Most of the previous studies examining kynurenines in HD used more readily available post-mortem brains from patients in the terminal stages of the disease (grades 3 and 4, as defined by Vonsattel *et al*., 1985). Increases in 3HKYN and KYNA were also observed in the cerebral cortex with the increase in 3HKYN, but not KYNA, being significant compared with control brains (Guidetti *et al*., 2000). The increases in 3HKYN were much greater than the increases in KYNA and when a ratio of 3HKYN: KYNA was examined, there was a significantly higher ratio with HD striatum compared with controls. To support this finding, 3HKYN and KYNA were measured in mice transgenic for full-length mutant huntingtin, which showed a similar degree of striatal neurodegeneration to that seen in grade 1 HD (Guidetti *et al*., 2000). Large increases in 3HKYN levels and smaller increases in KYNA concentrations were observed in the striatum and cortex, compared with control mice. Significant increases in the ratio of 3HKYN: KYNA were found in both the striatum and cortex, demonstrating an imbalance favouring the neurotoxin 3HKYN over the neuroprotectant KYNA at an early stage in the disease process. Beal *et al.* (1990) had also shown a significant increase in 3HKYN: KYNA ratio in HD post-mortem brains, but this was not recognised as important, as the levels of 3HKYN in HD brains were not significantly different from control brains. The authors put the emphasis on the increase in the ratio of kynurenine: KYNA, suggesting an impairment in the synthesis of KYNA.

For the first time, an increase in QUIN levels was demonstrated in HD brains, but only in post-mortem brains of grade 1 HD patients (Guidetti *et al.,* 2004). There were significant increases in 3HKYN and QUIN only in the early grade HD brains (grade 0/1) compared with control brains, in both the striatum and the cerebral cortex, but not in the cerebellum. In post-mortem brains from presymptomatic at-risk subjects, grade 2 HD, or late stage HD (grades 3/4), levels of 3HKYN and QUIN were not significantly different from control brains. There were no significant increases in the levels of kynurenine or KYNA at any stage of the disease or in any of the brain regions examined. When ratios of 3HKYN: KYNA and QUIN: KYNA were calculated, there were significant increases in both these ratios for early stage (grade 0/1) HD, but not for the at-risk individuals. These results suggest that 3HKYN and QUIN may participate in the initial phases of the neurodegenerative process in HD. The concentrations of either 3HKYN or QUIN observed by Guidetti *et al*. (2004) in grade 1 HD brain are similar to those known to induce neuronal loss *in vitro* (Chiarugi *et al*., 2001). As 3HKYN significantly potentiates QUIN neurotoxicity (Guidetti and Schwarcz, 1999), the increased levels of both metabolites may actually result in an elevated neuronal loss compared with that expected from the

concentrations of the individual compounds. Since free radicals can be generated by both QUIN (Rios and Santamaria, 1991) and 3HKYN (Vazquez *et al*., 2000), it is proposed that these free radicals potentiate NMDA receptor function and have a role in HD. The proposed hypothesis for kynurenine pathway involvement in HD is that 'increased levels of both 3HKYN and QUIN jointly accelerate neuronal death through NMDA receptor activation and the generation of toxic free radicals' (Guidetti *et al.,* 2004).

1.2.2.8 Glial Activation and Oxidative Stress in Huntington's Disease

An early participation of microglial cells in HD is suggested by abnormal microglial cytoarchitecture in the brain of grade 1 HD patients (Sapp *et al*., 2001). Stimulated microglia are far more efficient in generating 3HKYN than KYNA. Guidetti *et al.* (2004) suggested that the pathological process initiated by mutant huntingtin leads to an activation of glial kynurenine metabolism, where an increase in 3HKYN and QUIN, but not in KYNA, may accelerate neuronal damage.

There is evidence for a role of oxidative damage in HD. HD post-mortem brains have shown increased incidence of DNA strand breaks, excessive lipofuscin accumulation and increased immunohistochemical staining of oxidative damage products in HD striatum and cortex (Browne *et al*., 1999). The enhanced lipofuscin deposition in both striatal and cortical neurones in HD brain suggests that lipid peroxidation has occurred (Tellez-Nagel *et al*., 1974). Lipofuscin is a fluorophore which is produced by the reaction of amino compounds with aldehydic products of oxidative free radical-induced oxidation of, particularly, cell membrane lipids. The extent of lipofuscin accumulation increases with neuropathological severity of the disease. Free radical-induced oxidative damage was demonstrated by increased DNA strand breaks in HD striatal neurones (Butterworth *et al*., 1998). The degree of DNA fragmentation in HD striatum is positively correlated with the length of the polyglutamine expansion in huntingtin. Oxidative damage can also induce excessive oxidation of DNA bases, such as oxidising deoxyguanosine to 8-hydroxy-deoxyguanosine (8-OHDG). Significant increases in the levels of 8-OHDG were detected in nuclear DNA in the caudate of grade 4 HD post-mortem brains (Browne *et al.*, 1997), but not in other brain regions examined.

1.2.2.9 Therapeutic Approaches for Huntington's Disease

There is now much evidence for altered kynurenine pathway metabolism in HD, and several possible therapeutic approaches have been suggested. A possible therapy could be

to increase the levels of KYNA in the brain. Probenecid is a drug which inhibits the excretion of KYNA by blocking the transport system for organic acid disposal. Administration of probenecid in rats significantly increased the levels of KYNA in the brain (Vecsei *et al.,* 1992). However, when L-kynurenine, the natural bioprecursor of KYNA, was administered, the levels of KYNA also increased. There was a synergistic effect when both L-kynurenine and probenecid were administered, giving rise to over 50-fold increases in KYNA in both the cerebral cortex and the striatum. Administration of probenecid in humans with Parkinson's disease or Alzheimer's disease did significantly increase KYNA levels in the CSF (Vecsei *et al*., 1992). However, this strategy of increasing kynurenine or probenecid levels would also be expected to increase the levels of QUIN in the brain.

The use of kynurenine and probenecid, in conjunction with nicotinylalanine, an inhibitor of the enzymes, kyureninase and KMO, thus blocking QUIN synthesis, has been shown to attenuate QUIN-induced striatal neurotoxicity in rats, by increasing KYNA concentrations in the striatum (Harris *et al*., 1998). Other similar studies have been attempted for striatal neuroprotection involving enzymatic inhibition of 3HKYN and QUIN synthesis shifting kynurenine metabolism towards enhanced KYNA synthesis. Several KMO inhibitors have been developed and have shown neuroprotection in animal models (Speciale *et al.*, 1996; Cozzi *et al.*, 1999). Another mechanism of inhibiting QUIN synthesis is via the powerful glycine/NMDA receptor antagonist, 7-chlorokynurenic acid, which can be synthesised in the brain by the KAT enzymes following intracerebral injection of 4-chlorokynurenine (Wu *et al*., 1997). This novel approach has been suggested as a means of avoiding excessive receptor blockade and the associated clinical risks.

One successful treatment of HD is with dopamine antagonists which appear to relieve the choreic symptoms (Martin and Gusella, 1986). However, as the disease progresses, and the striatal cell loss increases, this relief becomes far less effective. HD is often associated with depression and severe mood swings, so drugs to alleviate these symptoms are often prescribed. One research approach to treatment involves the transplantation of human foetal striatal tissue to replace degenerated neurones in HD patients. Five patients received grafts of foetal tissue and three of the group showed improvements in motor and cognitive functions, with these benefits persisting for at least three years, suggesting a long-term effect (Freeman *et al*., 2000).

1.2.3 Stroke and Ischaemia

1.2.3.1 Ischaemic Stroke

Stroke is the third major cause of death in industrialised countries, with a mortality rate of about 30% (Dirnagl *et al*., 1999). Surviving stroke patients are often debilitated (including paralysis, speech problems and dementia, depending on the area of the brain that is damaged), and their care makes a significant contribution to health care costs. Most strokes (approximately 85%) are ischaemic (Green and Shuaib, 2006). Ischaemic stroke is caused by the blockage of a major artery in the brain which results in a decrease in blood flow and oxygen supply to the area of the brain around the blockage. The majority of human strokes results from an occlusion of the middle cerebral artery (Green and Shuaib, 2006), either by an embolus or by local thrombosis. Hence, most animal models of ischaemic stroke are based on a middle cerebral artery occlusion (MCAO) and can be transient or permanent.

Following a stroke, cells of the ischaemic core of the perfusion deficit are permanently damaged, with permanent depolarisation within minutes after the onset of ischaemia and cell death occurs rapidly (Dirnagl *et al*., 1999). The cells surrounding this core (ischaemic penumbra or peri-infarct zone) have low blood flow, with partially preserved energy metabolism, and have the potential to regain their function. After depolarisation, cells in the penumbra can repolarise but this uses energy and the cells may depolarise again in response to increased glutamate or potassium in the extracellular space. Hence, repetitive depolarisations (peri-infarct depolarisations) occur, which have been observed in animal models and can occur several times per hour and continue for at least 6-8 hours. After many depolarisations, the cells can no longer repolarise and the cells die, resulting in an increase in size of the ischaemic core or infarct (Mies *et al*., 1993). Many neurones in the penumbra can survive for many hours or even days after the ischaemic insult. Cells in the penumbra will ultimately die, thus expanding the ischaemic core, unless they are protected by reflow or by a neuroprotectant (Green and Shuaib, 2006).

MRI scans have shown that increasing ischaemic damage in stroke patients similarly occurs over a period of time (Baird *et al*., 1997). This suggests that neurological ability will also decrease over the same time. However, patients' symptoms tend to improve during the first week after the insult. This anomaly has raised the question of the importance of the delayed responses to the ischaemic insult in humans. However, this can be explained as the initial neurological deficit reflects the impairment of function in both

the core and the penumbra (Baird *et al.*, 1997). Over time, some parts of the penumbra may regain function, shown by an improvement in symptoms, while the structural lesion is actually still growing, as other cells of the penumbra die. It is not until after several days or weeks that the structural lesion size and location more closely reflects the neurological deficit. Long-term recovery of function may be explained by plasticity and tissue reorganisation (Dirnagl *et al.*, 1999).

There is a neurochemical sequence of events that occur following an ischaemic stroke. The major pathogenic mechanisms of this cascade are 1) excitotoxicity, 2) penumbra depolarisations, 3) inflammation and 4) programmed cell death (Dirnagl *et al.*, 1999).

1.2.3.2 Excitotoxicity after Ischaemia

Focal reduction of cerebral blood flow restricts oxygen and glucose availability for the cells and impairs the energetics required to maintain ionic gradients. This energy depletion causes neurones and glia to depolarise, voltage-dependent Ca^{2+} ion channels become activated and glutamate is released into the extracellular space. The reuptake of glutamate also requires energy and hence, upon energy failure, this lack of reuptake contributes to maintaining the high levels of extracellular glutamate. Glutamate receptors (NMDA and AMPA) are then activated, causing excitotoxicity, a major event for initiating ischaemic cell death (Dirnagl *et al.*, 1999). Activation of AMPA receptors causes sodium (Na^+) ions to enter the neurones and potassium (K^+) ions to be released into the extracellular space. Cell swelling occurs as the Na^+ ions influx causes water to passively enter the cells. The resulting brain oedema is one of the major determinants of whether the patient survives beyond the first few hours after stroke. Activation of NMDA receptors contributes to Ca^{2+} overload. Increased levels of Ca^{2+} ions have a large impact on tissue damage including the breakdown of several proteins and the production of reactive oxygen species causing lipid peroxidation of cell membranes.

Excitotoxicity can cause cells to die from necrosis, and can also initiate molecular events that lead to apoptosis. As a trigger for post-ischaemic inflammation, excitotoxicity can contribute to ischaemic injury by this mechanism. Excitotoxicity is therefore a major event for initiating ischaemic cell death, which has led to considerable work in this area for possible therapies for the treatment of stroke.

1.2.3.3 Oxidative Stress after Ischaemia

Reactive oxygen species also trigger inflammation and apoptosis. nNOS is a calcium-dependent enzyme and hence, with excessive Ca^{2+} ions, nitric oxide is synthesised which reacts with the superoxide anion to form the highly reactive peroxynitrite, causing further tissue damage. The importance of the role of nitric oxide after acute ischaemic stroke was shown in patients' CSF where increased levels of nitric oxide metabolites were associated with greater brain injury and early neurological deterioration (Castillo *et al*., 2000). Xanthine oxidase may also have a role in contributing to ischaemic brain damage, as following focal ischaemia in the rat, xanthine oxidase was shown to be involved in the generation of free radicals (Uemura *et al.*, 1991), although this has been debated (Betz *et al*., 1991). Iron-dependent free radical formation has been related to greater damage in cerebral ischaemia (Davalos *et al*., 2000) and therefore the presence of iron ions may be critical for oxidative damage in stroke. The antioxidant, melatonin, has been shown to suppress cerebral oedema caused by MCAO in rats (Torii *et al*., 2004). Mitochondria are a source of reactive oxygen species and when their membranes become impaired by free radical disruption, there is an oxygen free radical burst and cytochrome C is released, the latter providing a trigger for apoptosis (Kristian and Siesjo, 1998; Fujimura *et al.*, 1998).

1.2.3.4 Inflammation and Breakdown of the Blood-Brain Barrier after Ischaemia

The increased levels of Ca^{2+} ions, the increase in reactive oxygen species and the reduced oxygen availability all contribute to the rapid production of inflammatory mediators in the injured brain cells (Rothwell and Hopkins, 1995). Although inflammation is initiated very quickly following a stroke, it progresses as an inflammatory cascade over many hours or days (Barone and Feuerstein, 1999).

Post–ischaemic inflammation may contribute to ischaemic damage by many mechanisms including disruption of the blood-brain barrier (Skinner *et al*., 2006). Inflamed brain tissue activates endothelial and glial cells of the CNS which release inflammatory mediators, such as cytokines, causing increased permeability of the blood-brain barrier. This disruption of the blood-brain barrier allows leukocytes to infiltrate the CNS, causing the release of more inflammatory compounds including free radicals, resulting in further blood-brain barrier damage (Skinner *et al*., 2006). There was evidence of leukocyte accumulation as early as 6-12 hours after human stroke in partially perfused brain regions (Aspey *et al*., 1989) and at 30 minutes after permanent MCAO in rats. Leukocytes have

been hypothesised to be the cells responsible for the progression from tissue damage to cerebral infarction (Huang *et al*., 2006), as they trigger the production of more inflammatory mediators, which results in neutrophils, followed by macrophages and monocytes, migrating into the ischaemic brain, the latter two cell types becoming the predominant cells five to seven days after ischaemia (Iadecola, 1997).

Resident brain cells are also involved in the inflammatory response: microglia are activated and astrocytes become enlarged, four to six hours after ischaemia. Twenty-four hours after an MCAO, there is much microglial activation in the ischaemic brain, particularly in the penumbra (Dirnagl *et al*., 1999). Activated microglia can also produce neurotoxins including nitric oxide and reactive oxygen species contributing to further oxidative stress. The production of these neurotoxins reaches a peak at least several days after ischaemia, although the microglia are activated earlier (Giulian and Vaca, 1993). A recent study with human stroke patients showed little microglial activation until 72 hours after ischaemic stroke, with continued activation up to 30 days, assessed using a positron emission tomography (PET) technique (Price *et al*., 2006).

There is evidence that post-ischaemic inflammation contributes to cerebral damage, as this damage can be reduced when different steps in the process are blocked, such as blocking the action of the crucial inflammatory mediator IL-1 (Loddick and Rothwell, 1996). In stroke patients and in rodent models, infiltrating neutrophils produce iNOS which can generate toxic amounts of nitric oxide (Love, 1999). Pharmacological inhibitors of iNOS reduce ischaemic brain injury, even when administered a few days after the ischaemic insult, consistent with the hypothesis that ischaemic injury develops over several days (Iadecola, 1997). The cytokine, TNF-α, is produced by ischaemic neurones which can worsen the cerebral damage, but is also beneficial to the injured brain as it induces antioxidant enzymes (Skinner *et al*., 2006). Inflammation may also be linked with apoptosis, as reduced post-ischaemic inflammation also reduced apoptosis in the ischaemic brain (Chopp, 1996). In brain, as in other organs, inflammatory cells are involved in tissue reconstruction after injury, taking place days to weeks after ischaemia (Dirnagl *et al.*, 1999). Inflammation therefore, as well as being detrimental in exacerbating cerebral damage following a stroke, may also be crucial for tissue repair.

1.2.3.5 Cell Death after Ischaemia

Brain cells, damaged by excessive glutamate receptor activation, Ca^{2+} ion overload, oxygen radicals and mitochondrial and DNA damage, can die by either necrosis or

apoptosis. Necrosis is predominant in the ischaemic core, with apoptosis occurring within the penumbra. In ischaemia-mediated apoptosis, damaged mitochondria release cytochrome C resulting in activation of caspases. Caspases are protein-cleaving enzymes which act on many proteins, including the DNA-repairing enzyme PARP, the cytoskeletal protein, gelsolin, and huntingtin (Dirnagl *et al.*, 1999). Neurones seem to be particularly susceptible to caspase-mediated cell death following focal cerebral ischaemia. After mild reversible MCAO in mice, cytochrome C release and caspase effects are detected at 6 and 9 hours respectively, with cell death prominent between 24 and 72 hours (Endres *et al*., 1998). Caspase inhibitors reduce the volume of dead tissue in focal ischaemia and also decrease the neurological deficit (Hara *et al*., 1997), demonstrating the importance of caspase activity in reducing the functional ability of the neuronal tissue. Caspase inhibition also protects in experimental head trauma (Yakovlev *et al*., 1997). However, it has not been concluded that apoptosis definitely occurs in human ischaemic tissue.

1.2.3.6 Haemorrhagic Stroke

The other 15% of strokes are haemorrhagic where a blood vessel bursts either in the brain or on its surface (Green and Shuaib, 2006). Intracerebral haemorrhage produces a haematoma, a localised collection of clotted blood. Following acute haemorrhage, prevention of haematoma expansion is key for improving the outcome. However, there is a lack of proven treatments. As with ischaemic stroke, some perihaematoma tissue may be able to regain function, but much of the damage occurs quickly and irreversibly, with an explosion of local intracranial pressure at the site of the arteriolar rupture (Greenberg, 2007). As with ischaemic stroke, inflammation and cell death develop after a haemorrhage as shown with animal models of intracerebral haemorrhage (Wagner, 2007). In the perihaematoma tissue, there are infiltrating immune cells and activation of microglia. Elevated levels of glutamate have been measured in the brain after intracerebral haemorrhage (Wagner, 2007). When the haemorrhage occurs, blood glutamate can enter the brain's extracellular space and, as blood contains several fold higher concentrations of glutamate than the brain, may contribute to the increased glutamate in the brain. This increase in brain glutamate is expected to stimulate glutamate receptors and cause excitotoxicity. Oxidative stress has been shown to be rapidly induced in the perihaematomal white and grey matter after intracerebral haemorrhage. Oxidative stress contributed to DNA damage and brain injury in a rat model of intracerebral haemorrhage (Nakamura *et al*., 2005). Nakamura *et al*. (2005) also showed that intracerebral infusion of iron (Fe^{2+}) ions in rats increased markers of oxidative DNA damage supporting their hypothesis that iron chelation can reduce brain injury following intracerebral haemorrhage.

Blood-derived plasma proteins, in particular, thrombin, converts fibrinogen to fibrin, which is thought to lead to fibrin deposition and water retaining properties in the perihaematomal interstitial space (Wagner, 2007). This may contribute to the perihaematomal oedema. Pro-inflammatory cytokines are upregulated after intracerebral haemorrhage. Early upregulation of IL-1β has been observed in the perihaematoma, localised in astrocytes and microglial cells. This cytokine is known to increase blood-brain barrier permeability and increase oedema, and is upregulated in various brain injury models and in neurodegeneration (Wagner, 2007). Clot lysis using a recombinant tissue plasminogen activator (rt-PA) followed by surgical aspiration reduced the clot and oedema volumes and prevented disruption of the blood brain barrier in a pig intracerebral haemorrhage model. This treatment was shown to reduce cytokine expression. Early intervention using this approach is currently being examined in a clinical trial (Wagner, 2007). Iron chelation to reduce DNA oxidative damage has also been proposed as a potential therapy for intracerebral haemorrhage (Nakamura *et al*., 2005).

1.2.3.7 Kynurenines and Ischaemia

One potential mechanism for neuronal damage following ischaemia is an increase in the concentrations of QUIN in the brain. As IDO, the first enzyme of the kynurenine pathway, is stimulated by inflammation, the production of QUIN from tryptophan may not occur immediately following the ischaemic insult but only after the inflammatory response has been established. The activity of the QUIN-degrading enzyme, QPRT, remains low, even after brain injury in rats, allowing for the accumulation of QUIN (Speciale *et al*., 1987). In addition, QUIN's potency is related to the absence of effective removal mechanisms for extracellular QUIN (Foster *et al*., 1984b). Therefore, excessive QUIN production after ischaemia could lead to sustained activation of NMDA receptors and excitotoxicity.

Saito *et al*. (1993b) demonstrated increased QUIN levels and IDO activity in the brain at 4 days after transient global ischaemia in gerbils. Maximum QUIN levels and IDO activity were observed in the hippocampus, followed by the striatum, the regions where the neurones are most vulnerable to ischaemia. There were no increases in QUIN concentrations or IDO activity in the brain until 2 days after the ischaemic insult (Heyes and Nowak, 1990; Saito *et al*., 1993b), with levels starting to rise at 2 days, peaking at 4 days and being reduced by 7 days. The delayed increases in QUIN levels and IDO activity corresponded to the time when inflammation and local brain injury increased. QUIN levels in the CSF of these gerbils, although much lower than the levels in the brain tissue, were also increased at 4 days after ischaemia, but there were no changes in plasma.

Following ischaemia, the maximum brain levels of QUIN exceeded those in the blood (Saito *et al*., 1993b), suggesting that this increase in QUIN is not due to a breakdown of the blood brain barrier. Injection of labelled tryptophan into the brain was also shown to produce QUIN in the hippocampus of ischaemic gerbils which was not observed in control gerbils, indicating that the ischaemic hippocampus can produce QUIN from tryptophan, but the healthy brain cannot. The brain has low IDO activity compared to that of other tissues. In healthy rat brain tissue, no metabolism of tryptophan or kynurenine to produce QUIN has been determined (Speciale *et al*., 1989b). Labelled ANA did not produce QUIN in the ischaemic gerbil brain, indicating that this was not an alternative pathway for QUIN formation. However, 3HANA injected into the brain of control gerbils did produce QUIN in both the hippocampus and the cerebellum, demonstrating that there is sufficient 3HAO present to convert 3HANA to QUIN in the healthy brain (Saito *et al*., 1993b). It was demonstrated that as well as increases in IDO activity in the ischaemic hippocampus, there were significant increases in the activities of the other major enzymes of the pathway required for converting tryptophan to QUIN: namely KMO, kynureninase and 3HAO. In the ischaemic gerbil hippocampus, increases in kynureninase and 3HAO occurred 1 day post ischaemia, whereas there was no increase in KMO until day 2 and maximally at day 4, at the same time as the increases in IDO and QUIN were observed (Saito *et al*., 1993c). It may be that KMO, being localised in mitochondria, and not present in the cytosol of the brain, may suppress an increase in its activity in the brain. There was no change in KAT activity, which produces KYNA, between control and ischaemic gerbils.

Macrophages *in vitro* can convert tryptophan to QUIN, suggesting that these cells contain kynurenine pathway enzymes (Heyes *et al*., 1996). Macrophages were observed by immunocytochemistry in the gerbil hippocampus 4 days after ischaemia and to a lesser extent in the striatum, cortex and thalamus, consistent with the lower IDO and QUIN in these regions (Saito *et al*., 1993b). The production of QUIN from tryptophan in ischaemia appeared to require macrophage infiltrates in the lesioned brain area, resulting from the inflammatory response. It may be that macrophages are the source of kynurenine pathway enzymes to enable tryptophan to produce QUIN. Infiltrating macrophages from the blood contain IDO, KMO, kynureninase and 3HAO, and therefore increase KMO activity following an inflammatory response (Saito *et al*., 1993c). 3HAO is localised in astrocytes in healthy rat brain (Okuno *et al*., 1987), and therefore the increases in 3HAO may also be due to astroglial proliferation. However, it has been shown that there is increased 3HAO activity following excitotoxic lesions in the striatum of rats and in patients with HD, but there was no increase in QUIN in the brain. This may suggest that it is the increased availability of the substrate, 3HANA, rather than the activity of 3HAO which will

determine QUIN production, and 3HANA may be produced in activated macrophages. The concentrations of tryptophan were also significantly elevated in both the hippocampus and the CSF at 4 days after ischaemia in the gerbil. As there is more available substrate, this may enhance the production of QUIN.

1.2.3.8 Kynurenine 3-mono-oxygenase Inhibitors and Ischaemia

Increases in KYNA and decreases in 3HKYN and QUIN, brought about by the administration of KMO inhibitors, resulted in reduced ischaemic brain damage in animal models of ischaemia. Both KMO inhibitors, (*m*-nitrobenzoyl)-alanine and 3,4-dimethoxy-[-*N*-4-(nitrophenyl)thiazol-2yl]-benzenesulphonamide (Ro 61-8048) caused a dramatic reduction (greater than 80%) in the percentage of damaged pyramidal neurones in the hippocampal CA1 region of gerbils subjected to transient bilateral carotid occlusion (Cozzi *et al*., 1999). After MCAO in rats, these compounds also significantly reduced infarct volume by over 60% (Cozzi *et al*., 1999). The mechanisms of this neuroprotection were initially thought to be due to the increase in KYNA caused by inhibiting KMO. KYNA levels, measured by microdialysis, increased by approximately 25-fold in the ischaemic gerbil brain, to a maximum of 56nM, and by approximately 5-fold in the ischaemic rat brain, to a maximum of 85nM, under the experimental conditions (Cozzi *et al*., 1999). However, these levels of KYNA are much lower than KYNA concentrations able to offer functionality as an antagonist at NMDA receptors, as the minimum concentration required is approximately 30μM, when administered by microdialysis (Urenjak and Obrenovitch, 2000). Direct application of 100nM of KYNA into the gerbil hippocampus, which was then subjected to transient bilateral carotid occlusion, inhibited the increased glutamate output induced by ischaemia (Moroni *et al*., 2003), suggesting an alternative mechanism for KYNA's neuroprotective effects. NMDA receptor antagonists, as well as other glutamate receptor antagonists, are protective against ischaemic brain damage, and have been shown in the gerbil model by Pellegrini-Giampietro *et al.* (1994).

In an *in vitro* model of ischaemia, rat hippocampal slices, exposed to oxygen glucose deprivation (OGD) and the two KMO inhibitors, (*m*-nitrobenzoyl)-alanine and Ro 61-8048, reduced neuronal death and restored reduced KYNA concentrations (Carpenedo *et al*., 2002). However, KYNA itself at concentrations up to 30μM had no neuroprotective effect on these hippocampal slices. Levels of 100μM KYNA did offer neuroprotection which was attributed to antagonism at NMDA receptors (Carpenedo *et al*., 2002). The mechanism for the neuroprotection of the KMO inhibitors in ischaemia therefore was not simply by increased KYNA concentrations.

KMO inhibitors also inhibit the production of 3HKYN and QUIN synthesis and a reduction in these neurotoxic compounds may contribute to neuroprotection. Rat hippocampal slices were exposed to OGD in the presence of Ro 61-8048 and low concentrations (1-10μM) of either 3HKYN or QUIN inhibited the neuroprotective effect of Ro 61-8048 (Carpenedo *et al*., 2002). These results suggest that 3HKYN and QUIN contribute to the loss of hippocampal neurones after ischaemia. Indeed, a role for the reduced formation of 3HKYN in the mechanism for KMO inhibitors preventing ischaemic neuronal death has been further implicated as, in the *in vitro* OGD model of ischaemia, hippocampal slices demonstrated apoptotic features of neuronal death (Moroni *et al*., 2001) and 3HKYN is a potent apoptotic compound (Okuda *et al*., 1998; Chiarugi *et al*., 2001). The ability of KMO inhibitors to reduce ischaemic neuronal damage demonstrates that KMO inhibitors have the potential to be evaluated as therapeutic tools in the treatment of stroke.

The evidence above supports a role for IDO activation and the involvement of the kynurenine pathway in the mechanisms contributing to brain damage following ischaemic stroke. In particular, manipulating the levels of KYNA, 3HKYN and QUIN with KMO inhibitors has been shown to offer neuroprotection from ischaemic neuronal damage.

1.2.3.9 Treatment for Ischaemic Stroke

There are no effective treatments for acute ischaemic stroke, except for the thrombolytic rt-PA, which needs to be given within 3 hours of the onset of the stroke (Green and Shuaib, 2006). This drug, however, increases the risk of haemorrhage, which together with the need for immediate administration, results in it being given to less than 5% of stroke patients. Additionally, it is not always effective as many patients do not reperfuse when given the drug. There are two major approaches for the treatment of stroke: thrombolysis (which includes rt-PA) to try to disperse the clot and restore blood flow to the compromised region, and neuroprotection, using drugs to interfere with one or more of the mechanisms which are activated following ischaemia, to minimise the extent of tissue damage and hence neurodegeneration (Green and Shuaib, 2006). Several studies have examined a combination of thrombolytic and neuroprotective drugs but no synergistic effect was demonstrated. However, the potential neuroprotectant drug NXY-059 was shown to prevent the increase in haemorrhage rate produced by the thrombolytic drug, rt-PA (Lapchak *et al*., 2002). Aspirin, if started within 48hours of ischaemic stroke, has been shown to reduce the risk of early recurrent ischaemic stroke, without a major risk of haemorrhagic complications (Sandercock *et al*., 2003; Sherman, 2004). It also reduces the

volume of brain damage after a stroke, thus improving the long-term outcome for the patient. Aspirin inhibits the aggregation of blood platelets and hence enhances blood perfusion to the brain (Sandercock *et al*., 2003; Sherman, 2004).

1.2.3.10 Neuroprotective Therapy – NMDA Receptor Antagonists

There has been major progress in researching the mechanisms of ischaemic cell death and neuroprotective drugs but this has not translated into the development of suitable drugs for humans. This research has been based on rat and other animal models, with permanent MCAO and transient MCAO. During the 1990s, much animal work was done establishing excitotoxic glutamate receptor (NMDA and AMPA) antagonists which decreased the damage to the penumbra. In addition to KYNA itself, two competitive NMDA receptor antagonists, 2-amino-5-phosphonovalerate and 2-amino-7-phosphonoheptanoate, depressed the ischaemia-induced accumulation of glutamate and aspartate in the rat striatum (Ghribi *et al*., 1994). Since 2003, many pharmaceutical companies have pulled out of clinical trials with these neuroprotective drugs for stroke or head injury treatment, as they did not show therapeutic efficacy. Glutamate is released very early after the ischaemic insult and drugs need to be given very quickly which is often impractical (Green and Shuaib, 2006). For example, the NMDA receptor antagonist, MK-801, administered before or up to 2 hours after onset, reduced penumbra damage in a range of animal species (Park *et al*., 1988; McCulloch, 1992). Another approach was to antagonise the NMDA receptor by inhibiting glycine from its regulatory site, thus reducing NMDA receptor activity. Chen *et al*. (1993) had demonstrated that the potent glycine antagonist, 7-chlorothiokynurenic acid, attenuated infarct size in rats with permanent MCAO. Glutamate has a physiological role as a neurotransmitter and blocking glutamate receptors results in serious side effects such as respiratory depression and cardiovascular dysregulation (Dirnagl *et al*., 1999). To overcome this problem, recent trial drugs have examined targeting specific subunits of glutamate receptors that are upregulated in ischaemia. There appears to be only one NMDA receptor antagonist (Traxoprodil) currently being developed with NR2B receptor subtype selectivity (Green and Shuaib, 2006). Other clinical trials that have not produced the neuroprotection shown in animal models include administering magnesium (Mg^{2+} ions) to reduce activation of the NMDA receptor, as these ions 'gate' the NMDA receptor channel. AMPA antagonists were also evaluated as neuroprotectants, but competitive antagonists had adverse side effects (Green and Shuaib, 2006).

1.2.3.11 Failure of Neuroprotective Drugs in Clinical Trials

Reasons for the failure of neuroprotective drugs for treatment of stroke in humans although they worked well in animals, include a) poor penetration of drug from plasma to the brain, b) reduced dose to alleviate side effects to the cardiovascular system, c) uncontrolled physiology compared to animal work, e.g., blood pressure, temperature, glucose level, d) stroke patients are usually elderly, with a variety of other clinical problems whereas young healthy animals are used in animal models, e) penumbra may be smaller (unknown volume) and exist for a shorter period of time than in animals, f) may not have found the therapeutic window in human, as drugs were often given to humans at a much later time than they had been shown to be effective in animal models and g) may need to protect all brain cells including the axons and oligodendrocytes, neither of which contain NMDA receptor (Dirnagl *et al*., 1999; McCulloch and Dewar, 2001; Green and Shuaib, 2006). To maximise the chance of success of a drug, guidelines have been proposed by experts from academia and industry, the Stroke Therapy Academic Industry Roundtable group (STAIR), for the information that should be collected in animal models before a drug ought to be considered for progression to clinical trial (Stroke Therapy Academic Industry Roundtable, 1999).

1.2.3.12 Neuroprotective Therapy – Reduction in Oxidative Stress

NXY-059 is the first neuroprotective drug developed rigidly following the STAIR criteria before entering Phase III trials. The first of two clinical studies (SAINT I) with acute stroke patients has shown that this drug significantly reduced disability (using the modified Rankin score) but did not improve neurological function (measured by the US National Institutes of Health stroke scale). This drug was administered within 6 hours of stroke onset, and was well tolerated in the patients (Lees *et al*., 2006). The results of the second trial are awaited. NXY-059 is a nitrone which has free radical trapping properties. Its trapping ability means that it forms a stable adduct with free radicals, which may suggest that it is less reversible than some free radical scavengers. NXY-059 also prevents mitochondrial dysfunction by reducing cytochrome C release, and hence caspase activation, after the ischaemic insult (Green and Shuaib, 2006). Other free radical scavengers and trapping agents have been investigated. The most promising is stilbazulenyl nitrone (STAZN), another nitrone-derived compound, which recently produced effective neuroprotection in transient MCAO in the rat and has the potential benefit over NXY-059 in that it exhibits greater penetration of cerebral tissue (Ley *et al*., 2005). Another approach that is currently being considered for developing drugs for

ischaemic stroke is metal chelation, where compounds which chelate divalent metal ions, including calcium, copper and iron, have shown reduced infarct volumes in rat models, functioning in reducing free radical production. Metal chelation also has therapeutic potential in haemorrhagic stroke (Nakamura *et al*., 2005). Citicoline is a compound that has been in clinical use for many years in Europe and Japan to treat a variety of degenerative neurological disorders. It is currently being considered for the treatment of stroke as it reduces lipid metabolism following ischaemia and hence reduces the generation of free fatty acids and free radicals (Green and Shuaib, 2006).

1.2.3.13 Neuroprotective Therapy – Anti-inflammatory Approach

As inflammation contributes to cerebral damage following a stroke, there is a strong rationale to use anti-inflammatory therapies (Dirnagl *et al*., 1999). The production of cytokines, for example TNF-α, is important in the inflammatory response of the brain to injury. Anti-TNF-α antibodies have been shown to inhibit ischaemia-induced damage to the brain in spontaneously hypertensive rats (Barone *et al*., 1997) and several approaches to reduce this TNF-α effect have been examined. The major problem with reducing TNF-α is that this compound also can assist in cell survival after stroke, therefore, it has both beneficial and detrimental properties. Other stroke therapies involve growth factors, which may be more important for long-term recovery than for neuroprotection (Green and Shuaib, 2006).

1.2.4 Traumatic Brain Injury

TBI is a closed head injury which occurs when there is a sudden blunt mechanical impact injury to the head, causing disruption of the brain tissue. The leading causes of TBI are motor vehicle crashes and falls, with a mortality rate of 22%. TBI is most common in young people, in the age range 15-24, and in the elderly, over 75 years old, and is more prevalent in males than females (Lovasik *et al*., 2001).

The primary injury from a TBI occurs from both the direct contact force and the resulting translational or rotational acceleration forces in the brain which immediately follow the initial impact (Lovasik *et al*., 2001). There is immediate damage to neurones and glia which lose their functionality. Damage to the cerebral vasculature leads to progressive events including increased intracranial pressure, disruption of the blood-brain barrier, haemorrhage, ischaemia, oedema and necrosis (Stelmasiak *et al*., 2000). As well as focal injury leading to large lesions in the brain, a TBI involving rotational acceleration causes

diffuse axonal injury, a scattered destruction of axons throughout the brain (Sahuquillo *et al*., 2001). This diffuse axonal injury is the most devastating primary lesion. Mechanical alteration of axons and cells leads to increased permeability and abnormal ion fluxes with increases in intracellular calcium, sodium and chloride ions and leakage of potassium ions, resulting in excitotoxicity and astrocytic swelling (Sahuquillo *et al*., 2001). After diffuse axonal injury, reduced levels of free magnesium ions have been found, which could contribute to increased excitotoxicity as magnesium ions block the NMDA receptor channels.

Following TBI, ruptured cerebral blood vessels allow the blood contents to escape directly into the brain parenchyma, which is thought to contribute to secondary brain damage. The permeability of the blood-brain barrier in a mouse model of TBI showed that soon after trauma, both large and small molecules could enter the brain near the injury site (Habgood *et al*., 2007). Large protein-sized molecules could no longer penetrate the blood-brain barrier by 4-5 hours after injury, however, much smaller molecules (<10,000Da) could still reach the brain tissue up to 4 days post-injury. This demonstrated that the restoration of the blood-brain barrier was gradual and penetration during this restoration period was dependent on the size of the molecules in the blood (Habgood *et al*., 2007). TBI has been shown to alter polyamine metabolism with activation of NMDA receptors via their polyamine sites, which contributes to blood-brain barrier breakdown, brain oedema and injury volume (Dempsey *et al*., 2000).

Traumatic brain lesions are very dynamic and the mechanical injury activates multiple neurochemical cascades. As in stroke, after TBI, there are areas of injured but viable brain tissue known as the traumatic penumbra, which, for some time after the initial injury, have the potential to regain their function (Sahuquillo *et al*., 2001). Secondary damage occurs at variable times after the initial injury and mechanisms include oxidative stress, excitotoxicity and inflammation. Macrophages and microglia have been reported to be involved in the inflammatory damage after TBI, releasing oxygen free radicals and cytokines (Sahuquillo *et al*., 2001). For patients undergoing surgery within 24 hours after trauma, a biopsy of contused human brain tissue was removed and a very limited inflammatory response was detected. However, brain tissue from TBI patients having surgery 3-5 days after trauma showed a massive inflammatory response, involving macrophages and reactive microglia (Holmin *et al*., 1998). Similarly, the inflammatory marker, neopterin, has been shown to gradually increase in CSF and serum during the first week after human TBI (Lenzlinger *et al*., 2001).

One of the major secondary effects of TBI is ischaemia. TBI-induced ischaemia can be caused by decreases in global cerebral blood flow due to increased intracranial pressure or reduced blood flow due to vasospasm or brain herniation (where the arteries are compressed by the herniated tissue) (Sahuquillo *et al*., 2001). Although the causes of ischaemia are different compared with ischaemic stroke, the resulting effects are the same: excitotoxicity, oedema, oxidative stress and inflammation. Ischaemic damage has been the main therapeutic target for both experimental and clinical trials for TBI and many of the pharmacological agents trialled for TBI were similar to those used in ischaemic stroke trials. As for stroke, clinical trials with drugs that were very promising in experimental animal models of TBI have yielded disappointing results, suggesting that for head-injured patients, neuroprotection cannot be achieved as simply as in animal models (Sahuquillo *et al*., 2001).

1.2.4.1 Kynurenines in Traumatic Brain Injury

The involvement of excitotoxicity and inflammation in TBI suggests that the kynurenine pathway may be involved in the resulting brain damage. Sinz *et al*. (1998) determined there was a rise in QUIN in the CSF during the first week after severe TBI in humans with QUIN levels being higher in patients who died as a result of their injury. QUIN concentrations in the CSF increased over time, reaching a maximum, averaging 463nM, at 72-83 hours after the TBI, compared to normal concentrations of QUIN in the CSF of less than 50nM. The time course of this increase mirrors that of infiltration of macrophages into the injured brain (Holmin *et al*., 1997). Children with severe TBI also showed high levels of QUIN in their CSF, which increased over time after injury and was higher in children who died (Bell *et al*., 1999).

In animal studies, a traumatic compression injury to the spinal cord of guinea pigs resulted in increased QUIN levels in the spinal cord and CSF and caused progressive neurological deficits in the animals (Blight *et al*., 1995). This study showed that peak tissue levels of QUIN occurred at 12 days after injury. The 3HAO inhibitor, 4-chloro-3-hydroxyanthranilate, reduced QUIN accumulation and reduced the functional deficits, demonstrating QUIN's role in causing neuronal dysfunction. Further work showed that this improvement in the guinea pigs' neurological function was not caused by the 3HAO inhibitor resulting in an increase in the neuroprotectant, KYNA, as there was no change in the concentration of this metabolite (Yates *et al*., 2006).

With a rat model of TBI (lateral fluid-percussion brain injury), the neuroprotective effects of KYNA were demonstrated, with KYNA administered 15 minutes post-injury. KYNA dramatically reduced the trauma-induced cognitive dysfunction, improved neurologic motor deficits, reduced oedema formation and attenuated calcium concentrations in the rat brain (Smith *et al*., 1993). This model causes the selective loss of neurones in the hippocampal CA3 region, observed after two weeks, which was greatly reduced by the KYNA treatment (Hicks *et al*., 1994).

1.3 Aims

The literature suggests that the kynurenine pathway may have a role in HD, stroke and TBI, contributing to the neuronal damage in each of these disorders.

The initial aim of this PhD project was to determine whether concentrations of kynurenines are altered in the blood of patients with different causes of brain injury: HD, chronic brain injury and acute stroke. The project then aimed to investigate the kinetics of tryptophan metabolism via the kynurenine pathway following tryptophan depletion or loading for HD and chronic brain injury patients compared with healthy controls. For stroke patients, the project aimed to determine any changes in the kynurenines during the 14 days following the stroke.

The overall aim was to hypothesise mechanisms for changes in kynurenines in each disorder and compare kynurenine pathway metabolism in neurodegeneration (HD), chronic brain injury and acute brain injury (stroke).

This project also aimed to develop high performance liquid chromatography (HPLC) methods to accurately measure kynurenines in human blood.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Sigma-Aldrich Company Ltd., Poole, Dorset, UK: L-tryptophan (T-0254), L-kynurenine (K-8625), kynurenic acid (K-3375), 3-hydroxy-DL-kynurenine (H-1771), 3-hydroxyanthranilic acid (H-9391), anthranilic acid (A8,985-5), 3-nitro-L-tyrosine (N-7389), L-ascorbic acid, ethylenediaminetetraacetic acid disodium salt (EDTA), 3,4-dihydroxybenzylamine hydrobromide, 4-amino-3-hydroxybenzoic acid.

VWR International Ltd., Lutterworth, Leicestershire, UK: glacial acetic acid (100%), orthophosphoric acid (85%), perchloric acid (70%), sodium acetate trihydrate, sodium chloride, triethylamine (HiPerSolv for HPLC), zinc acetate, potassium hydroxide pellets, disodium hydrogen orthophosphate (anhydrous), sodium dihydrogen orthophosphate dihydrate.

Fisher Scientific UK, Loughborough, Leicestershire, UK: citric acid, 1-heptane sulphonic acid (sodium salt, HPLC grade), hydrochloric acid (37%), sodium hydroxide pellets, HPLC grade acetonitrile, HPLC grade methanol.

Rathburn Chemical Company, Walkerburn, Scotland, UK: HPLC grade water.

Millipore (UK) Limited, Watford, Hertfordshire, UK: Milli-Q synthesis system producing ultrapure water for HPLC, fed by the Millipore Elix 10 water system. Water purity was 18.2mΩ.

BOC Gases, Guildford, Surrey, UK: helium gas, oxygen-free nitrogen gas.

All chemicals were stored at the appropriate temperature, as recommended by the suppliers.

2.1.2 Laboratory Consumables

General laboratory consumables (eppendorf tubes, pipette tips, pasteur pipettes, universal and bijou containers, glassware) were obtained from a variety of sources based on

availability in the University Biomedical Research (IBLS) Stores. Autosampler total recovery vials were purchased from Waters Limited (Elstree, Hertfordshire, UK). Polypropylene pellet pestles (for homogenising residues in 1.5ml eppendorf tubes) were from VWR.

2.1.3 Filtration of Solutions

All HPLC mobile phases were filtered under vacuum through 0.2µm nylon filter membranes (Phenomenex, 47mm diameter), using filtration glassware from Phenomenex. All solutions prior to injection on to the HPLC were filtered by centrifuging using 0.2µm VectaSpin Micro Anopore filter tubes (Whatman International, Maidstone, Kent, UK) or 0.2µm PVDF Micro-Spin filter tubes (Alltech Associates Applied Science Ltd., Carnforth, Lancashire, UK).

2.1.4 Laboratory Equipment

Sanyo, Loughborough, Leicestershire, UK: Harrier 18/80, MSB080.CR2.K refrigerated centrifuge, with a 24 x 1.5ml angle rotor (43117-612).

Thermo Electron Corporation, Milford, MA, USA: Modulyo freeze-dryer.

2.1.5 HPLC Instrumentation

The HPLC system was from Waters Ltd., consisting of a Waters 600 quaternary pump, a Waters 717plus autosampler, a column heater, a Waters 2487 dual wavelength absorbance detector and a Waters 474 fluorescence detector. Flow was through the absorbance detector followed by the fluorescence detector, the detectors being connected in series. Alternatively a Waters Concorde electrochemical detector was used instead of the absorbance and fluorescence detectors. Waters Millennium software (version 3.20) was used to run the HPLC system, collect the data and process the chromatograms. Helium gas was used to degas all mobile phases continuously at a sparge rate of 20ml/min.

2.1.6 HPLC Columns

The HPLC columns used were Kingsorb, a standard C18 column (250mm x 4.6mm I.D., particle size 5µm,) and Synergi Hydro-RP 80A C18 column (250mm x 4.6mm I.D., particle size 4µm) both from Phenomenex, Macclesfield, Cheshire, UK. The Synergi

Hydro column contains a C18 bonded phase endcapped with a polar group to provide good retention of both hydrophobic and polar compounds with 100% aqueous mobile phase.

Phenomenex SecurityGuard Cartridges (4 x 3.0mm) were used as pre-columns, the general C18 cartridge with the Kingsorb column and the polar endcapped C18 cartridge for the Synergi Hydro column.

2.1.7 Solid Phase Extraction (SPE)

A 24-position vacuum manifold (Phenomenex) was used for SPE. Oxygen-free nitrogen gas was used for drying samples in organic solvents and a Modulyo freeze-dryer was used for drying aqueous samples.

Various SPE cartridges were assessed: Waters SepPak (500mg/3cc), Waters SepPak (200mg/3ml), Waters Oasis HLB (30mg/1ml), Phenomenex Strata (2g/12ml), Phenomenex Strata-X (30mg/1ml), Varian (Walton-on-Thames, Surrey, UK) Focus (20mg/3ml), Phenomenex Strata-X-C (60mg/3ml), Varian Bond Elut SCX (500mg/3ml), Varian Bond Elut SCX (50mg/1ml), Phenomenex Strata-X-C (30mg/1ml), Supelco (Sigma-Aldrich) Discovery DSC-MCAX (100mg/1ml) and Waters Oasis MCX (30mg/1ml).

2.2 HPLC Technique

Reverse phase HPLC was used in this project. It is a commonly used type of liquid chromatography, where separation of an analyte (or analytes) in a solution containing several different compounds is achieved based on its polarity. The HPLC columns are often C18 columns where the column matrix, usually silica particles, are coated with straight hydrocarbon chains containing 18 carbon atoms. The mobile phase is relatively polar in comparison, usually comprising an aqueous buffered salt solution, with the addition of a small amount of organic solvent. When the sample is injected on to the column, the more non-polar components interact with the column packing material and are retained for longer by the column, whereas the more polar compounds pass easily through the column and are eluted more quickly. On elution of the analyte in the mobile phase it passes through a detector which produces a signal, the intensity of which corresponds to the quantity of analyte present. The detector used depends on the properties of the analyte, with absorbance detectors commonly used at ultraviolet and visible wavelengths and fluorescence and electrochemical detectors are often used for increased sensitivity, when the analytes can fluoresce, or are redox-active. Mass spectrometry detection has been used

more recently, with this detection system having the added advantage of confirming the identity of the analyte as it is detected.

2.3 Methods (carried out by others)

Quinolinic acid was analysed using gas chromatography-mass spectrometry (GC-MS) at West Park Biochemistry Laboratories, Epsom General Hospital, Epsom, Surrey, UK and the standard clinical assays, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), were analysed by the Haematology and Immunology Laboratories, Epsom General Hospital, Epsom, Surrey. In addition, neopterin and lipid peroxidation products were detemined by Dr C.M. Forrest and Ms L. Oxford at the University of Glasgow.

Method details are given in the Appendix.

3 HPLC Method Development

3.1 Sample Preparation

3.1.1 Method Development for Sample Preparation

A simple extraction procedure using perchloric acid (PCA) to deproteinise the plasma samples was examined, based on the extraction described by Hervé *et al*. (1996).

A single extraction released only a proportion of the kynurenines from plasma, and as the levels of some kynurenines in plasma are in the low nanomolar range and difficult to detect, it was important to try to extract all of the kynurenines present. Repeated extractions were examined to try to extract more kynurenines.

The first extraction procedure used a minimum amount of extraction solvent (total volume of 700µl). In a 1.5ml eppendorf tube, 500µl plasma was added to 100µl 240µM 3-nitrotyrosine (internal standard) and 50µl 2mM ascorbic acid and the mixture was vortexed. 50µl 4M PCA was added and vortexed immediately for 30 seconds. The samples were centrifuged at 5000g for 10 minutes at 4°C and the resulting supernatant frozen overnight at -40°C.

Another procedure involved a single extraction again with extracts being prepared as above, except that a larger volume of extraction solution (total volume of 1ml) was used. 200µl water was added to the plasma and the volume of 4M PCA was increased to 150µl, compared with the original 50µl.

To release kynurenines from plasma using a repeated extraction method, the first extraction procedure was used and after collecting the supernatant from the centrifugation step, the residue was then homogenised with 200µl water and 100µl 4M PCA, using pellet pestles. This mixture was vortexed and then centrifuged at 5000g for 10 minutes at 4°C. The resulting supernatant was combined with the initial supernatant.

A method involving three extractions was also examined by washing the residue produced on centrifugation twice. The method from the repeated extraction procedure was followed using volumes for the first wash of 100µl water and 50µl 4M PCA, with a repeated

washing step using a further 100µl water and 50µl 4M PCA. The three resulting supernatants were combined.

The total volumes for the two and three extraction methods were both 1ml. The following day extracts were defrosted and filtered through Anopore filter tubes by centrifuging at 10,000g for 10 minutes at 4°C.

Four plasma samples were used to determine the best extraction procedure for measuring tryptophan, kynurenine and KYNA. Tryptophan, kynurenine and KYNA were determined by the HPLC method described in Section 3.2.3. The concentration in plasma was determined by multiplying the results from the calibration curve by 2, where the plasma had been diluted from 500µl into a total volume of 1ml. The results from the calibration curve for the concentrated extracts have been multiplied by 700/500 as 500µl plasma was diluted in a total volume of 700µl. The internal standard or recovery factors have not been applied to these results as it is the actual quantities of the individual metabolites that are important here. The results are shown in Tables 3-1 to 3-3.

Another four plasma samples were extracted by the four different extraction methods detailed above and the amounts of ANA and 3HANA were determined by the method described in Section 3.3.3. The results are in Tables 3-4 and 3-5.

Four plasma samples taken 7 hours after subjects had consumed a tryptophan load were extracted using the same four extraction procedures. The results for the determination of 3HKYN using the method described in Section 3.5.3 are shown in Table 3-6.

In general, the more concentrated extract and the other extract from a single extraction procedure resulted in lower concentrations of all six metabolites compared with the repeated extractions. For kynurenine and ANA, the concentrated extracts were lower than when a larger volume of extraction solvent was used in a single extraction. The different volumes of extraction solvent showed little effect for the other metabolites. Increasing the number of extractions in general increased the levels of all the metabolites, although there was only a small improvement with three extractions compared with two. Kynurenine seemed to be the least affected by the number of extractions. The results suggest that increasing the number of extractions beyond three would not extract much more of the metabolites and hence the three extraction procedure was chosen for use throughout this work.

The kynurenines, 3HKYN and 3HANA, are unstable and light sensitive, but can be stabilised by the addition of the antioxidant, ascorbic acid (Pearson and Reynolds, 1991). Ascorbic acid was added to standard solutions and during extraction of plasma samples to maintain a concentration of 100µM in all solutions, to try to prevent breakdown of these compounds. All standards and plasma samples were defrosted in the dark and kept on ice once defrosted.

3.1.2 Final Sample Preparation Method

Samples of EDTA plasma were defrosted in the dark and kept on ice during sample preparation. To 100µl 240µM 3-nitrotyrosine (internal standard), 500µl plasma and 50µl 2mM ascorbic acid were added, followed by 50µl 4M PCA. Samples were vortexed for 30 seconds immediately after acid addition, centrifuged at 5000g for 10 minutes at 4°C, and the supernatant collected. The precipitated proteins were homogenised with 100µl water and 50µl 4M PCA using pellet pestles, the mixture vortexed for 30 seconds and centrifuged at 5000g for 10 minutes at 4°C. Again the supernatant was collected. This washing and centrifugation step was repeated and the supernatants combined. After vortexing, the extracts were split into two samples and stored at -40°C prior to analysis.

Defrosted sample supernatants were filtered through Anopore or Alltech filter tubes by centrifugation at 10,000g for 10 minutes at 4°C. Filtrates were transferred to Waters total recovery vials and placed in the autosampler at 4°C. A volume of 100µl was injected on to the HPLC column.

Table 3-1 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of tryptophan in four plasma samples.

	Tryptophan (μM)			
	Plasma 1	Plasma 2	Plasma 3	Plasma 4
One extraction (conc)	20.7	51.2	38.7	34.4
One extraction	20.5	52.1	38.8	34.5
Two extractions	23.6	59.2	44.9	39.7
Three extractions	24.6	60.7	45.6	40.7

Table 3-2 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of kynurenine in four plasma samples.

	Kynurenine (μM)			
	Plasma 1	Plasma 2	Plasma 3	Plasma 4
One extraction (conc)	1.43	1.69	1.24	1.16
One extraction	1.61	2.12	1.47	1.31
Two extractions	1.73	2.10	1.51	1.38
Three extractions	1.71	2.17	1.61	1.38

Table 3-3 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of kynurenic acid in four plasma samples.

	Kynurenic Acid (nM)			
	Plasma 1	Plasma 2	Plasma 3	Plasma 4
One extraction (conc)	44.4	22.0	16.8	25.1
One extraction	41.6	26.2	17.9	21.9
Two extractions	57.8	28.9	19.0	28.0
Three extractions	56.0	28.6	22.3	29.6

Table 3-4 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of anthranilic acid in four plasma samples.

	Anthranilic Acid (nM)			
	Plasma 5	Plasma 6	Plasma 7	Plasma 8
One extraction (conc)	26.6	111.4	12.6	12.2
One extraction	40.0	118.9	19.7	18.4
Two extractions	47.6	132.5	19.9	18.2
Three extractions	52.2	136.5	20.3	20.8

Table 3-5 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of 3-hydroxyanthranilic acid in four plasma samples (analysed using HPLC with fluorescence detection).

	3-Hydroxyanthranilic Acid (nM)			
	Plasma 5	Plasma 6	Plasma 7	Plasma 8
One extraction (conc)	18.0	0	20.8	23.7
One extraction	18.7	0.9	20.5	24.2
Two extractions	17.4	1.4	23.1	26.7
Three extractions	25.3	0.9	23.5	27.4

Table 3-6 Effect of increasing the volume of extraction solvent and increasing the number of extractions on the levels of 3-hydroxykynurenine in four plasma samples.

	3-Hydroxykynurenine (μM)			
	Plasma 9	Plasma 10	Plasma 11	Plasma 12
One extraction (conc)	1.08	0.75	0.70	0.60
One extraction	1.12	0.64	0.69	0.57
Two extractions	1.21	0.76	0.73	0.67
Three extractions	1.30	0.78	0.74	0.68

3.2 Quantification of Tryptophan, Kynurenine and KYNA

3.2.1 Determination of Tryptophan, Kynurenine and KYNA Introduction

Tryptophan and kynurenine have been determined in human serum (Hervé *et al.*, 1996) Kynurenine was detected by absorbance at wavelength 365nm, and tryptophan by either absorbance at 230nm or fluorescence at excitation wavelength (λ_{ex}) of 254nm and emission wavelength (λ_{em}) of 404nm. Widner *et al*. (1997) also measured tryptophan and kynurenine in human serum, using 3-nitrotyrosine as the internal standard. Kynurenine and 3-nitrotyrosine were determined by absorbance at 360nm. Tryptophan was detected using fluorescence with λ_{ex}=285nm and λ_{em}=365 nm. The use of 3-nitrotyrosine is not ideal as an internal standard as it can be produced in the body under conditions of oxidative stress. Widner *et al.* (1997) used 3-nitrotyrosine in samples at a concentration of 45µM, which exceeds pathophysiological amounts by at least two orders of magnitude, and hence 3-nitrotyrosine was regarded suitable for use as an internal standard.

The concentration of KYNA in biological fluids is low and a sensitive method of detection is required. Endogenous concentrations of KYNA in the serum of healthy humans are between 23nM and 59.6nM (Heyes *et al*., 1994; Hervé *et al*., 1996; Ilzecka *et al*., 2003; Fukushima *et al*., 2007). This concentration is not detectable using absorbance or fluorescence detection, nor is it electrochemically active, therefore electrochemical detection is not appropriate. However, Shibata (1988) demonstrated that KYNA could be detected by enhancing its fluorescence with zinc ions (Zn^{2+}). KYNA was separated using reverse phase HPLC, with up to 1M zinc acetate post-column derivatisation, resulting in a 50-fold increase in the fluorescence of KYNA. The fluorescence λ_{ex} was 344nm and λ_{em} was 398nm. Hervé *et al*. (1996) incorporated zinc acetate into the mobile phase at a concentration of 250mM and successfully measured KYNA in human serum. This group also measured tryptophan and kynurenine in the same run but using absorbance detection, showing that the zinc acetate does not interfere with the detection of these metabolites.

3.2.2 Method Development for Tryptophan, Kynurenine and KYNA

The mobile phase chosen was based on that of Hervé *et al*. (1996) and comprised 50mM acetic acid, 250mM zinc acetate and 5% acetonitrile, with kynurenine, tryptophan, 3-

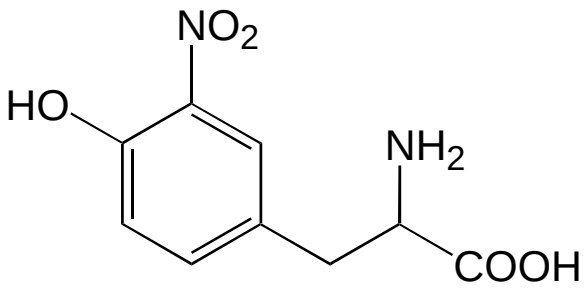
nitrotyrosine and KYNA all eluting within 20 minutes. However, with the large number of samples involved in each study, continuous running of this mobile phase was impractical as the HPLC pump frequently blocked due to precipitation of zinc acetate. The best compromise was using a mobile phase composed of 50mM acetic acid, 100mM zinc acetate and 3% acetonitrile, which resulted in a retention times of 10.2 minutes for kynurenine, 16.2 minutes for 3-nitrotyrosine, 20.5 minutes for tryptophan and 25 minutes for KYNA using the Kingsorb HPLC column. The standard C18 Kingsorb column is not ideal for use with mobile phases containing less than 5% organic modifier, as the C18 phase may collapse, and therefore it was replaced by a Synergi Hydro HPLC column, containing a C18 bonded phase endcapped with a polar group, which can be used with completely aqueous mobile phases. When the column was changed to the Synergi Hydro column for these measurements, the acetonitrile concentration was reduced to 1.5% to produce similar chromatograms, with retention times of 10.2 minutes for kynurenine, 16.4 minutes for 3-nitrotyrosine, 20.7 minutes for tryptophan and 29.6 minutes for KYNA. The method was run isocratically as running a gradient would involve increasing the concentration of the organic modifier, acetonitrile, which may cause zinc acetate precipitation in the pump.

Patients who had high lipid peroxidation results were compared with controls to check that they did not produce detectable levels of 3-nitrotyrosine, which would interfere with the 3-nitrotyrosine internal standard peak on the chromatograms. Two patients with extremely high levels of lipid peroxidation products (11.6μM and 12.3μM), one from the HD study and the other from the chronic brain injury study were compared with two patients with high lipid peroxidation in the stroke study (lipid peroxidation 1.28μM and 1.79μM) and two stroke controls (lipid peroxidation 0.20μM and 0.21μM). Samples were prepared by the standard extraction method (Section 3.1.2), using 100μl water instead of 100μl 3-nitrotyrosine. Samples were analysed using the 100mM zinc acetate mobile phase. There were no peaks for 3-nitrotyrosine in any of the samples assessed. The limit of detection (LoD) of 3-nitrotyrosine was 100nM, with an injection volume of 100μl. As no 3-nitrotyrosine could be detected in the samples, 3-nitrotyrosine was considered to be a suitable internal standard for this analysis.

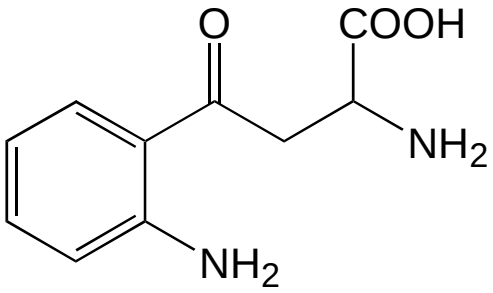
The concentration of internal standard, 3-nitrotyrosine, used was 24μM, as this gave a large peak at both 250nm and 365nm, which could be analysed on the same scale as peaks for the metabolites being measured. 3-Nitrotyrosine peak height was comparable with the peak height for 40μM tryptophan and 12μM kynurenine. The structure of 3-nitrotyrosine is shown in Figure 3-1 and has some similarities with the structure of kynurenine. It was

assumed that any losses in metabolites were similar to losses in 3-nitrotyrosine on extraction.

Standard solutions of 3HKYN, 3HANA and ANA could also be measured with this mobile phase. However, with plasma samples, there were other peaks interfering with peaks for 3HANA and ANA, even when the fluorescence wavelengths were optimised to λ_{ex}=320nm, λ_{em}=420nm for these compounds. For 3HKYN standards, the retention time was 5.4 minutes, which in plasma samples, interfered with lots of compounds eluting at about the same retention time and also the LoD was high with only concentrations of above 100nM able to be detected at either 250nm or 365nm. 3HKYN concentrations in healthy human serum have been reported as 32nM (Pearson and Reynolds, 1991). In summary, it was not possible to quantify 3HANA, ANA or 3HKYN in human blood samples using this mobile phase.



3-nitrotyrosine
MW = 226.2



kynurenine
MW = 208.2

Figure 3-1 Comparison of the structure of 3-nitrotyrosine (internal standard) with the structure of kynurenine.

(Courtesy of TW Stone)

3.2.3 Final Method for the Determination of Tryptophan, Kynurenine and KYNA

Isocratic reverse phase HPLC was performed at 37°C. Separation was achieved using a Kingsorb C18 column or a Synergi Hydro C18 column and the detection system included both the dual wavelength absorbance detector (250nm and 365nm) and the fluorescence detector, connected in series. The mobile phase, pumped at a flow rate of 1ml/min, was 50mM acetic acid, 100mM zinc acetate containing 3% acetonitrile. The pH of the zinc acetate buffer was 4.7. With the Synergi Hydro column the concentration of acetonitrile was reduced to 1.5%. Tryptophan was determined by absorbance detection at a wavelength of 250nm and kynurenine and 3-nitrotyrosine were detected at 365nm. KYNA was determined by fluorescence detection (λ_{ex}=344nm, λ_{em}=390nm).

A volume of 100µl of filtered sample extract or standard was injected on to the HPLC.

Calibration curves were analysed with the samples at least twice weekly when running continuously. A new calibration curve was run after any shutdown of the HPLC. A standard solution was also analysed after each block of 10 runs to check for any drift in the retention times of the metabolites. A blank, containing only water, ascorbic acid and PCA was run with each calibration curve to ensure the baseline was completely flat at the retention times for the peaks.

Example chromatograms are shown in Figure 3-2.

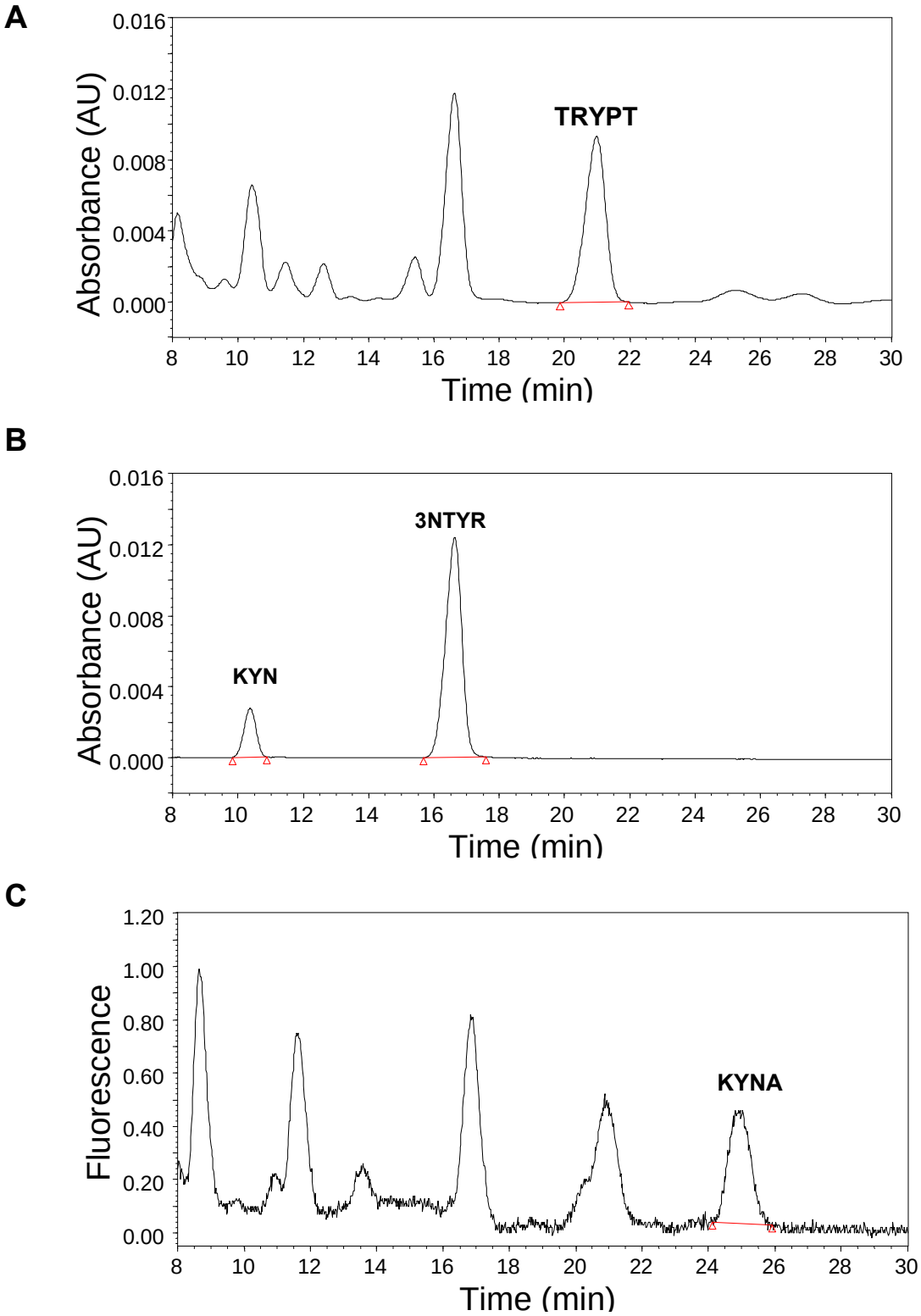


Figure 3-2 Example chromatograms of an extracted plasma sample from a patient showing peaks for (A) tryptophan at 250nm, (B) kynurenine and 3-nitrotyrosine at 365nm and (C) kynurenic acid by fluorescence at λ_{ex}=344nm, λ_{em}=390nm.

3.2.4 Preparation of Tryptophan, Kynurenine and KYNA Standards

2mM tryptophan and 1mM kynurenine solutions were prepared from the pure powders by dissolving in HPLC grade water. KYNA required sodium hydroxide to solubilise and to prepare 1mM KYNA, the pure powder was dissolved in 10mM sodium hydroxide solution. KYNA was further diluted in HPLC grade water, by serial dilutions of 1 in 10, to achieve 10µM. 1ml aliquots of 2mM tryptophan, 1mM kynurenine and both 1mM and 10µM KYNA were frozen at -40°C. For the stroke study, a mixed standard solution 1 was prepared by defrosting these individual standard solutions (using 10µM KYNA) and diluting with HPLC grade water to produce a solution of 200µM tryptophan, 8µM kynurenine and 100nM KYNA. This mixed standard solution was also aliquoted into volumes of 1ml and frozen at -40°C until required to prepare a standard curve. Standard solutions were kept for up to 12 months at -40°C. Stability of these standard solutions at -40°C was examined and is discussed in Section 3.4.

A 240µM solution of the internal standard, 3-nitrotyrosine, was prepared by solubilising in 10mM hydrochloric acid, prepared using HPLC grade water. This solution was frozen in aliquots of 100µl in 1.5ml eppendorf tubes, which were then used directly for sample or standard preparation. These internal standard aliquots were also kept at -40°C for up to 12 months.

3.2.5 Calibration Curves for Tryptophan, Kynurenine and KYNA

To prepare a calibration curve for the stroke study, 2ml mixed standard 1 and 8 aliquots of 3-nitrotyrosine were defrosted. The mixed standard 1 was diluted 1 in 5 with HPLC grade water to prepare mixed standard 2 (40µM tryptophan, 1.6µM kynurenine and 20nM KYNA). Various volumes of mixed standard and water were added to each of the eppendorfs containing 3-nitrotyrosine (Table 3-7). 50µl of 2mM ascorbic acid and 150µl of PCA were added, to ensure their compositions were comparable with the sample extracts. A blank was also prepared, containing water, ascorbic acid and PCA only. The resulting concentrations of the 8 standard solutions are shown in Table 3-8.

For the HD and chronic brain injury studies, samples were analysed after tryptophan depletion and loading, and hence calibration curves over a much wider range of concentrations were required. A mixed standard (3) of 800µM tryptophan, 200µM kynurenine and 20µM KYNA was prepared from the frozen individual standards. This

was serially diluted tenfold to produce mixed standard 4 (80µM tryptophan, 20µM kynurenine and 2µM KYNA), mixed standard 5 (8µM tryptophan, 2µM kynurenine and 200nM KYNA) and mixed standard 6 (0.8µM tryptophan, 0.2µM kynurenine and 20nM KYNA). These mixed standards were used to prepare 14 standards of varying concentrations, each being mixed with HPLC grade water, 240µM 3-nitrotyrosine, 2mM ascorbic acid and 4M PCA as shown in Table 3-9. The resulting concentrations of the standards are shown in Table 3-10.

As with the plasma extracts, the standard solutions of various concentrations and the blank were filtered through Anopore or Alltech filter tubes using centrifugation at 10,000g for 10 minutes at 4°C. Filtrates were transferred to Waters total recovery vials and placed in the autosampler at 4°C. A volume of 100µl was injected on to the HPLC column.

Calibration curves were prepared from chromatogram peak areas of these mixed standard solutions. For kynurenine, determined at the absorbance wavelength of 365nm, the internal standard was included in the calibration curve and in quantifying the samples. This compensates for any loss in recovery of the metabolites during sample extraction. For absorbance detection at 250nm and fluorescence detection, quantified results were later corrected for recovery, using a recovery factor calculated from the area of 3-nitrotyrosine at 365nm in each extract. The mean area of 3-nitrotyrosine in the 8 standards was divided by the area of 3-nitrotyrosine in each extract. This gives a recovery factor which can be multiplied by the result for tryptophan and KYNA to compensate for any losses on extraction. This recovery factor showed more than 100% recovery. This was due to a concentration effect, as after protein precipitation and washing the precipitate, the volume of extract containing the kynurenines was approximately 800µl, although calculations are based on a volume of 1ml. Actually the recovery factor and the use of the internal standard calibration accounts for the losses on extraction and the concentration effects for each extract.

The results for the plasma extracts from the calibration curves were multiplied by 2 to give the concentration in the original plasma sample and then multiplied by the recovery factor for that sample, to produce the final concentration of tryptophan and KYNA in the plasma samples. For kynurenine, the results from the calibration curves were multiplied by 2 only, as the internal standard is included in the calibration.

Table 3-7 Preparation of standard solutions for calibrations curves of tryptophan, kynurenine and kynurenic acid for the stroke study.

Standard Code	Mixed Std	Volume Mixed Std (μl)	Volume Water (μl)	Volume 2mM Ascorbic Acid (μl)	Volume IS (μl)	Volume PCA (μl)
Std1	2	200	500	50	100	150
Std2	2	300	400	50	100	150
Std3	2	400	300	50	100	150
Std4	1	100	600	50	100	150
Std5	1	200	500	50	100	150
Std6	1	300	400	50	100	150
Std7	1	400	300	50	100	150
Std8	1	500	200	50	100	150
BLANK		0	800	50	0	150

Standard (Std), 240μM 3-nitrotyrosine internal standard (IS), 4M perchloric acid (PCA)

Table 3-8 Concentrations of tryptophan, kynurenine, kynurenic acid and 3-nitrotyrosine in standards for calibration curves for the stroke study.

	Concentration			
	Tryptophan (μM)	**Kynurenine (μM)**	**Kynurenic Acid (nM)**	**3-Nitrotyrosine (μM)**
Std1	8	0.32	4	24
Std2	12	0.48	6	24
Std3	16	0.64	8	24
Std4	20	0.8	10	24
Std5	40	1.6	20	24
Std6	60	2.4	30	24
Std7	80	3.2	40	24
Std8	100	4	50	24

Table 3-9 Preparation of standard solutions for calibrations curves of tryptophan, kynurenine and kynurenic acid for the Huntington's disease and chronic brain injury studies.

Standard Code	Mixed Std	Volume Mixed Std (μl)	Volume Water (μl)	Volume 2mM Ascorbic Acid (μl)	Volume IS (μl)	Volume PCA (μl)
Std1	6	100	600	50	100	150
Std2	6	250	450	50	100	150
Std3	6	500	200	50	100	150
Std4	5	100	600	50	100	150
Std5	5	250	450	50	100	150
Std6	5	500	200	50	100	150
Std7	4	100	600	50	100	150
Std8	4	200	500	50	100	150
Std9	4	400	300	50	100	150
Std10	4	500	200	50	100	150
Std11	3	100	600	50	100	150
Std12	3	250	450	50	100	150
Std13	3	400	300	50	100	150
Std14	3	500	200	50	100	150
BLANK		0	800	50	0	150

Standard (Std), 240μM 3-nitrotyrosine internal standard (IS), 4M perchloric acid (PCA)

Table 3-10 Concentrations of tryptophan, kynurenine, kynurenic acid and 3-nitrotyrosine in standards for calibration curves for the Huntington's disease and chronic brain injury studies.

	Concentration			
	Tryptophan (μM)	**Kynurenine (μM)**	**Kynurenic Acid (nM)**	**3-Nitrotyrosine (μM)**
Std1	0.08 not detectable	0.02 not detectable	2	24
Std2	0.2	0.05	5	24
Std3	0.4	0.1	10	24
Std4	0.8	0.2	20	24
Std5	2	0.5	50	24
Std6	4	1	100	24
Std7	8	2	200	24
Std8	16	4	400	24
Std9	32	8	800	24
Std10	40	10	1,000	24
Std11	80	20	2,000	24
Std12	200	50	5,000	24
Std13	320	80	8,000	24
Std14	400	100	10,000	24

3.2.6 Method Validation for the HPLC Method for Tryptophan, Kynurenine and KYNA

3.2.6.1 Linearity of HPLC Response for Tryptophan, Kynurenine and KYNA

Tryptophan, kynurenine and KYNA standards all demonstrated a linear HPLC response with increasing concentrations. This was observed across the full range of concentrations used in the calibration curves from 0.2µM to 400µM for tryptophan, from 0.05µM to 100µM for kynurenine and from 2nM to 10,000nM for KYNA.

3.2.6.2 Limit of Detection (LoD) for Tryptophan, Kynurenine and KYNA

The LoD is the smallest amount of the analyte that can be identified as a peak on the chromatogram, distinguishable from the baseline, defined as a signal/noise ratio of 3.

Dilutions of standard solutions of tryptophan, kynurenine and KYNA were analysed to determine the LoD for standards, using the injection volume of 100µl. The LoD for this method was 0.2µM (20pmoles on column) for tryptophan, 0.03µM (3pmoles on column) for kynurenine and 1nM (0.1pmoles on column) for KYNA. When the Kingsorb HPLC column was replaced with the Synergi Hydro column, the LoD was the same for tryptophan and kynurenine, but was 2nM (0.2pmoles on column) for KYNA, probably due to the later elution time.

Tryptophan depleted samples from the HD and chronic brain injury studies were used to determine the LoD for kynurenine and KYNA in plasma samples. The LoD for kynurenine in plasma was 0.1µM, and for KYNA was 4nM, accounting for the plasma being diluted 2-fold for this method and with an injection volume of 100µl. The LoD for tryptophan in plasma could not be determined as even the tryptophan depleted samples had tryptophan levels that were easily detectable, with the lowest level measured being 3.2µM.

3.2.6.3 Recoveries for Tryptophan, Kynurenine and KYNA

To ensure that no metabolite was lost during the extraction process, which is not accounted for by the use of the internal standard (or recovery factor calculated from it), a spike of mixed standard solution was added to a plasma sample before extraction. Consistent

recoveries demonstrate the accuracy of the method. Three plasma samples were used, each spiked at three different concentrations of a mixed standard (Table 3-11).

Samples were extracted using the standard method described in Section 3.1.2, with the exceptions that 20µl of spike solution was added to the plasma before extraction, and the volume in which the precipitated proteins were resuspended was changed to 90µl water and 50µl 4M PCA for each wash.

The percentage recovery for each spike is calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Conc. of Spiked Plasma} - \text{Conc. of Original Plasma}}{\text{Conc. of Standard Spike}} \times 100\%$$

Recoveries for all three metabolites ranged from 90% to 109% (Table 3-12), showing that the method is accurate, with the results giving a true measure of the quantity of tryptophan, kynurenine and KYNA present. The internal standard or recovery factor calculated from the internal standard peak was used to adjust samples for recoveries as described in Section 3.2.5 and not the percentage recoveries calculated here.

3.2.6.4 Intra-Assay and Inter-Assay Precision for Tryptophan, Kynurenine and KYNA

To determine intra-assay precision or repeatability, a sample of serum was pooled from the stroke control subjects. 8 aliquots of pooled serum were extracted by the standard method (Section 3.1.2). After freezing the extracts overnight at -40°C, all 8 extracts were analysed by HPLC. Tryptophan concentrations were 51.7 ± 0.87 µM (mean ± standard deviation (SD)) with CV = 1.7%. The mean concentration of kynurenine was 2.09 ± 0.05 µM, CV = 2.5%. KYNA concentrations were 36.6 ± 2.7 nM, CV = 7.4%.

For inter-assay precision or reproducibility, a sample was pooled from the control serum for the stroke study. 8 samples were extracted by the standard method and the extracts frozen at -40°C, at least overnight. One inter-assay sample was analysed each day for the following 8 days together with other samples being analysed. This involved 3 different calibration curves being used to calculate the results. The mean concentration of tryptophan was 54.6 ± 0.66 µM, CV = 1.2%, kynurenine was 2.09 ± 0.06 µM, CV = 2.9%, and KYNA was 41.9 ± 2.12 nM, CV = 5.1%.

The coefficient of variation (CV) is calculated by dividing the standard deviation by the mean and expressing the result as a percentage. A CV of below 10% for inter-assay variability was acceptable for these HPLC analyses.

3.2.6.5 Confirmation of Peak Identity for Tryptophan, Kynurenine and KYNA

HPLC with absorbance and fluorescence detection produce chromatographic peaks which are identified as the analyte by retention time alone. It is possible that another compound may be present in the sample which elutes at the same time as the analyte of interest. The detector absorbance wavelength or the fluorescence wavelengths may be specific enough for only the analyte of interest to be detected, however, it is possible the other compound could also be detected and be mistaken as the analyte, leading to erroneous concentrations of the analyte being calculated. An advantage of using a mass spectrometer as the detector with HPLC or GC is that the chromatographic peaks are specifically identified as the analyte of interest.

To ensure that the peaks measured are pure metabolite, tryptophan, kynurenine and KYNA were detected by 2 different detection methods, as it is highly unlikely that another compound would give the same response by both methods. Tryptophan can be detected at the fluorescence wavelengths used for KYNA detection (λ_{ex}=344nm, λ_{em}=390nm). Kynurenine can be measured by absorbance at 250nm, although there is often interference with the kynurenine peak from other compounds at this wavelength. It is difficult to determine the low levels of KYNA in plasma using alternative detection, but KYNA in the tryptophan loaded samples were detectable with absorbance at 250nm. Results for 6 samples of each metabolite analysed by the different detection methods are shown in Table 3-13.

The average percentage change in the results using the alternative detection was 5% for tryptophan, 6% for kynurenine and 9% for KYNA. Tryptophan and KYNA results from the alternative method were sometimes lower and sometimes higher than the standard results, but all 6 kynurenine results were higher by the alternative method. This may suggest that even in these kynurenine samples where there was minimal interference, there may still be a little interference at 250nm. This clarified that the peaks being measured were indeed tryptophan, kynurenine and KYNA.

Table 3-11 Concentrations of standard solutions of tryptophan, kynurenine and kynurenic acid (KYNA) used to spike plasma to determine recoveries.

		Spike conc. for 20μl spike	Conc. of spike as if in plasma
Tryptophan (μM)	Low level	500	20
	Medium level	1000	40
	High level	1500	60
Kynurenine (μM)	Low level	20	0.8
	Medium level	40	1.6
	High level	80	3.2
KYNA (nM)	Low level	500	20
	Medium level	1000	40
	High level	1500	60

Table 3-12 Recoveries for tryptophan, kynurenine and kynurenic acid (KYNA), using 3 different plasma samples spiked with 3 concentrations of each metabolite.

	Spike Conc as if in plasma	Plasma 1		Plasma 2		Plasma 3	
		Conc in plasma	Recovery (%)	Conc in plasma	Recovery (%)	Conc in plasma	Recovery (%)
Tryptophan (μM)		59.1		53.1		57.2	
	20	78.0	94	72.6	98	75.2	98
	40	95.1	90	90.1	93	94.0	92
	60	117.0	96	110.1	95	116.3	90
Kynurenine (μM)		2.46		2.29		2.04	
	0.8	3.24	98	3.08	98	2.87	104
	1.6	3.91	91	3.96	104	3.68	102
	3.2	5.65	100	5.24	92	5.40	105
KYNA (nM)		38.3		39.2		41.6	
	20	60.2	109	58.9	99	61.8	101
	40	75.4	93	76.4	93	79.3	94
	60	96.3	97	94.3	92	95.7	90

Table 3-13 Measurement of tryptophan, kynurenine and kynurenic acid (KYNA) using an alternative method to check that the peaks identified are indeed pure metabolite.

Plasma Sample	Tryptophan (μM)		Plasma Sample	Kynurenine (μM)		Plasma Sample*	KYNA (μM)	
	standard method 250nm	FL		standard method 365nm	absorbance 250nm		standard method FL	absorbance 250nm
1	61.5	58.9	**7**	2.99	3.15	**13**	1.37	1.25
2	75.4	81.7	**8**	1.80	1.91	**14**	2.32	2.33
3	61.4	65.5	**9**	2.86	3.19	**15**	0.70	0.64
4	57.2	58.9	**10**	1.88	1.88	**16**	4.03	4.38
5	22.5	22.8	**11**	2.26	2.36	**17**	0.80	0.90
6	44.5	42.2	**12**	3.44	3.73	**18**	1.60	1.82

standard method 250nm or 365nm is with absorbance detection
FL – fluorescence at excitation 344nm/ emission 390nm
* - plasma samples taken after tryptophan loading

3.3 Measurement of 3HANA and ANA by fluorescence detection

3.3.1 Determination of 3HANA and ANA Introduction

For the HD and chronic brain injury studies, 3HANA was measured using electrochemical detection (Section 3.5), but the sensitivity was low, with levels only just detectable in baseline control and patient bloods (LoD was 40nM for plasma samples). A method which increased the sensitivity of 3HANA was sought. The method by Cannazza *et al*., 2003, describes quantification of 3HANA and ANA in rat brain dialysate by reverse phase HPLC with fluorescence detection. This method used a mobile phase consisting of 25mM sodium acetate/acetic acid buffer at pH 5.5 and 10% methanol. The mobile phase used for fluorescence detection of KYNA is 100mM zinc acetate/ 50mM acetic acid buffer at pH 4.7 and 1.5% acetonitrile (Section 3.2.3). The sensitivity of 3HANA with these two mobile phases was compared. For fluorescence detection of 3HANA and ANA, several references had suggested the optimum λ_{ex} of approximately 320nm and λ_{em} of approximately 420nm. (Cannazza *et al*., 2003; Hervé *et al*., 1996; Chiarugi *et al*., 1996). These wavelengths were compared with the wavelengths used for KYNA measurement (λ_{ex}=344nm, λ_{em}=390nm). It would also be useful to determine ANA in the same run, as there was interference with this peak using the zinc acetate mobile phase.

3.3.2 Method Development for 3HANA and ANA

A sodium acetate mobile phase was prepared using 25mM sodium acetate trihydrate with the addition of acetic acid until the pH reached 5.5. Using standards of 2μM, and 40nM 3HANA (just detectable by electrochemical detection), a comparison of zinc acetate mobile phase pH 4.7 (Section 3.2.3) without acetonitrile, with sodium acetate mobile phase pH 5.5, without methanol was made. 40nM 3HANA was detected using both mobile phases, but the sodium acetate mobile phase produced a much larger peak. All measurements were made using fluorescence detection (λ_{ex}=320nm, λ_{em}=420nm).

The electrochemical method for measuring 3HANA used a mobile phase at pH 3.1. The retention time was 18 minutes. The sodium acetate mobile phase for the fluorescence method had a pH of 5.5, which gave a retention time of 10.3 minutes. A reason for this change may be that at the higher pH, 3HANA will be in an ionised form, as opposed to being non-ionised, and hence be retained on the column for a shorter time.

The 40nM 3HANA standard was measured at λ_{ex}=320nm, λ_{em}=420nm and at λ_{ex}=344nm, λ_{em}=390nm, the fluorescence wavelengths used for KYNA measurement. The former wavelengths produced a larger peak than the latter.

The sodium acetate mobile phase with fluorescence detection at λ_{ex}=320nm, λ_{em}=420nm was the most sensitive method for measuring 3HANA.

To ensure that endogenous 3-nitrotyrosine could not be detected in the plasma samples using this mobile phase, samples with high lipid peroxidation and controls were assessed as described previously (Section 3.2.2). No 3-nitrotyrosine could be detected in any of the samples. The LoD for 3-nitrotyrosine was 100nM using this sodium acetate mobile phase. The use of 24μM 3-nitrotyrosine was suitable as the internal standard.

3.3.3 Final Method for the Determination of 3HANA and ANA

Using a Synergi Hydro column with isocratic reverse phase HPLC at 30°C, 3HANA and ANA were measured by fluorescence detection at λ_{ex}=320nm, λ_{em}=420nm. The internal standard, 3-nitrotyrosine, was determined by the absorbance detector at 365nm, which was connected in series with the fluorescence detector. The mobile phase of 25mM sodium acetate buffer (containing 2mM acetic acid) at pH5.5, containing no organic modifier was pumped at a flow rate of 1ml/min.

The injection volume of filtered sample extract or standard solution was 100μl.

Calibration curves were analysed with the samples at least twice weekly when running continuously. A new calibration curve was run after any shutdown of the HPLC. A blank, containing only water, ascorbic acid and PCA was run with each calibration curve to ensure the baseline was completely flat at the retention times for the peaks. A standard was run through the HPLC after every 10 samples, to check for any drift in the retention times of the metabolites.

An example chromatogram is shown in Figure 3-3.

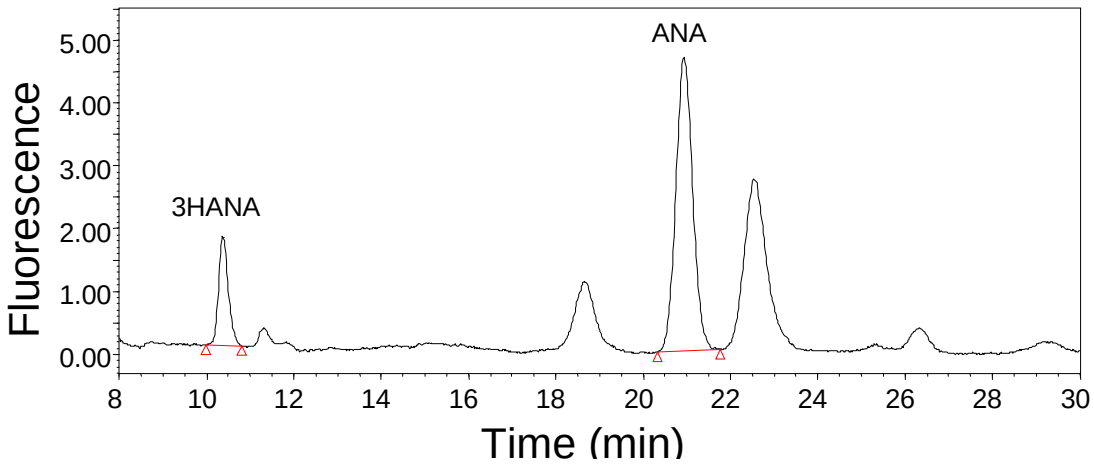


Figure 3-3 Example chromatogram of an extracted serum sample from a patient showing peaks for 3-hydroxyanthranilic acid and anthranilic acid by fluorescence detection at λ_{ex}=320nm, λ_{em}=420nm.

3.3.4 Preparation of 3HANA and ANA standards

3HANA and ANA would not solubilise in water alone, requiring hydrochloric acid and sodium hydroxide, respectively, to enable solubilisation. 2mM 3HANA was prepared from the pure powder by dissolving in 40mM hydrochloric acid and 2mM ANA was prepared using 10mM sodium hydroxide. As 3HANA is an unstable compound which may autoxidise, the hydrochloric acid and the sodium hydroxide solutions both were prepared using 100µM ascorbic acid (in HPLC grade water) as an antioxidant. These stock standard solutions were divided into 1ml aliquots, which were frozen at -40°C for up to 12 months. The stability of these standard solutions in the freezer was assessed and the results discussed in Section 3.4.

3.3.5 Calibration Curves for 3HANA and ANA

To prepare a calibration curve for the stroke study, 2mM 3HANA and 2mM ANA were defrosted, as were 8 x 100µl aliquots of 240µM 3-nitrotyrosine (prepared as described in Section 3.2.4). 3HANA and ANA were mixed and diluted by serial dilutions, using 100µM ascorbic acid throughout, to produce mixed standard 1 (200nM 3HANA and 800nM ANA), mixed standard 2 (40nM 3HANA and 160nM ANA) and mixed standard 3 (5nM 3HANA and 20nM ANA). Various volumes of these mixed standards were added to the internal standard, 3-nitrotyrosine, and diluted with 100µM ascorbic acid. 4M PCA was added to each of these standards, and extra ascorbic acid was added to compensate for the 3-nitrotyrosine and PCA not containing ascorbic acid, to maintain a concentration of 100µM ascorbic acid in the final solutions. A blank of ascorbic acid and PCA was also prepared. The preparation of standards and their final concentrations are shown in Tables 3-14 and 3-15.

The standards and the blank were filtered through Anopore or Alltech filter tubes using centrifugation at 10,000g for 10 minutes at 4°C. Filtrates were transferred to autosampler vials and maintained in the autosampler at 4°C. A volume of 100µl was injected on to the HPLC column.

Calibration curves were prepared from chromatogram peak heights, as the peak for ANA in plasma often had slight interference from an adjacent peak, so peak areas would not be accurate. 3HANA and ANA were determined by fluorescence detection. At the absorbance wavelength of 365nm, the mean area for the internal standard 3-nitrotyrosine from the 8 standards was used to calculate a recovery factor for each sample from its area

of 3-nitrotyrosine, as described in Section 3.2.5. The results for the plasma extracts from the calibration curves were multiplied by 2 to give the concentration in the original plasma sample and then multiplied by the recovery factor for that sample, to produce the final concentration of 3HANA and ANA in the plasma samples.

Table 3-14 Preparation of standard solutions for calibrations curves of 3-hydroxyanthranilic acid and anthranilic acid for the stroke study.

Standard Code	Mixed Std	Volume Mixed Std (μl)	Volume 100μM Ascorbic Acid (μl)	Volume 600μM Ascorbic Acid (μl)	Volume IS (μl)	Volume PCA (μl)
Std1	3	100	600	50	100	150
Std2	3	400	300	50	100	150
Std3	2	100	600	50	100	150
Std4	2	150	550	50	100	150
Std5	2	200	500	50	100	150
Std6	2	300	400	50	100	150
Std7	2	400	300	50	100	150
Std8	1	100	600	50	100	150
BLANK		0	800	50	0	150

Standard (Std), 240μM 3-nitrotyrosine internal standard (IS), 4M perchloric acid (PCA)

Table 3-15 Concentrations of 3-hydroxyanthranilic acid and anthranilic acid and 3-nitrotyrosine in standards for calibration curves for the stroke study.

	Concentration		
	3-Hydroxyanthranilic Acid (nM)	**Anthranilic Acid (nM)**	**3-Nitrotyrosine (μM)**
Std1	0.5	2	24
Std2	2	8	24
Std3	4	16	24
Std4	6	24	24
Std5	8	32	24
Std6	12	48	24
Std7	16	64	24
Std8	20	80	24

3.3.6 Method Validation for the HPLC Method for 3HANA and ANA

3.3.6.1 Linearity of HPLC Response for 3HANA and ANA

3HANA and ANA standards showed a linear HPLC response with increasing concentrations. This was observed across the range of concentrations in the calibration curves from 0.5nM to 20nM for 3HANA and from 2nM to 80nM for ANA.

3.3.6.2 LoD for 3HANA and ANA

Standard solutions of 3HANA and ANA were diluted to determine the LoD for these standards, with a signal/noise ratio of 3. The LoD for 3HANA standard solution was 0.1nM (10fmoles on column), for an injection volume of 100µl. The LoD for ANA was 0.2nM, again for an injection volume of 100µl, which equates to 20fmols on column.

In the stroke study, where this method was used, as both 3HANA and ANA were easily detected in serum samples, a LoD for serum samples for this method could not be determined. Levels as low as 0.75nM 3HANA and 8.9nM ANA in serum were detectable.

3.3.6.3 Recoveries for 3HANA and ANA

Three samples of serum from healthy controls for the stroke study were spiked with mixed standard solutions of 3HANA and ANA. All three samples were spiked at three different concentrations of 3HANA and ANA, prior to extraction, and were compared with the three original unspiked samples.

The method for preparing the samples and the calculation for recovery was as described in Section 3.2.6.3.

Mixed standard solutions of 3HANA and ANA were prepared by defrosting and diluting stock solutions of 2mM individual standards and mixing them to give the appropriate concentrations of each standard for a 20µl spike, for each level of spike (Table 3-16). All standard solutions were prepared in 100µM ascorbic acid. The concentration of plasma was determined in the usual way, multiplying the result from the calibration curve by 2, and by the recovery factor.

The recoveries varied from 92% to 107% for 3HANA and from 96% to 107% for ANA (Table 3-17), demonstrating that this method of extraction, with the subsequent analysis, accurately quantifies the levels of 3HANA and ANA.

Table 3-16 Concentrations of standard solutions of 3-hydroxyanthranilic acid and anthranilic acid used to spike plasma to determine recoveries.

		Spike conc. for 20μl spike (nM)	Conc. of spike if had been in plasma (nM)
3-Hydroxyanthranilic Acid	Low level	250	10
	Medium level	500	20
	High level	1000	40
Anthranilic Acid	Low level	1000	40
	Medium level	2000	80
	High level	4000	160

Table 3-17 Recoveries of 3-hydroxyanthranilic acid (3HANA) and anthranilic acid (ANA) using 3 different plasma samples spiked with 3 concentrations of each metabolite.

	Spike Conc as if in plasma (nM)	Plasma 1		Plasma 2		Plasma 3	
		Conc in plasma (nM)	Recovery (%)	Conc in plasma (nM)	Recovery (%)	Conc in plasma (nM)	Recovery (%)
3HANA		5.4		15.1		11.8	
	10	15.3	100	25.5	104	21.0	92
	20	25.3	100	36.4	107	31.9	101
	40	46.4	102	56.4	103	52.9	103
ANA		24.7		26.8		24.4	
	40	67.5	107	67.2	101	62.8	96
	80	109.1	106	107.6	101	104.5	100
	160	190.3	104	188.8	101	188.2	102

3.3.6.4 Intra-Assay and Inter-Assay Precision for 3HANA and ANA

Several plasma samples from healthy controls for the stroke study were pooled to produce a sample for repeatability. The sample was extracted 8 times by the standard procedure (Section 3.1.2), and extracts were frozen at -40°C. 8 extracts were defrosted and analysed together in the same run, to obtain a measure of repeatability. The concentrations of 3HANA were 34.1 ± 0.81 nM (mean ± SD), with CV = 2.4%, and for ANA, the mean concentration was 29.9 ± 1.7 nM, CV = 5.5%.

Similarly, plasma samples from healthy controls for the stroke study were pooled for a sample for reproducibility. 8 aliquots were extracted on the same day and frozen at -40°C. One sample was then analysed each day, together with other samples being analysed on that day, for the following 8 days. These 8 repeats were analysed using 3 different calibration curves. The concentrations for 3HANA were 24.5 ± 2.2 nM (mean ± SD), with CV = 9.0%, and for ANA, the mean concentration was 26.3 ± 2.1 nM, CV = 8.1%.

3.3.6.5 Confirmation of Peak Identity for 3HANA and ANA

To clarify that the 3HANA peak identified by this method was indeed pure 3HANA, 6 plasma samples were also measured using the electrochemical mobile phase with electrochemical detection (Section 3.5). The results are shown in Table 3-18. The mean % change in the results was 8% for 3HANA. The electrochemical method produced some higher and some lower results than the original as expected due to normal variability. Concentrations determined were similar by either method, confirming that the peaks were pure 3HANA.

ANA can also be determined using the zinc acetate mobile phase method (Section 3.2.3) with the fluorescence wavelengths changed to those optimised for ANA (λ_{ex}=320nm, λ_{em}=420nm). The results for six plasma samples analysed by both the original and this alternative method are shown in Table 3-18. The mean % change in the results is 7%. Both methods gave similar results, confirming that the peaks were pure ANA.

Table 3-18 Measurement of 3-hydroxyanthranilic acid and anthranilic acid using an alternative method to check that the peaks identified are indeed pure metabolite.

Plasma Sample	**3-Hydroxyanthranilic Acid (nM)**		**Plasma Sample**	**Anthranilic Acid (nM)**	
	standard method FL	ECD +0.65V		standard method FL	FL zinc acetate mobile phase
1	149	151	**7**	20.2	16.6
2	57	50	**8**	28.3	26.1
3	48	47	**9**	23.8	26.7
4	1351	1452	**10**	41.4	39.5
5	57	46	**11**	42.4	42.5
6	134	125	**12**	78.9	77.3

FL – fluorescence at excitation 320nm/ emission 420nm
ECD – electrochemical detection

3.4 Stability of Standard Solutions and Plasma Samples Stored at -40ºC

3.4.1 Storage of Standard Solutions at -40ºC

It would be ideal if standard solutions of tryptophan, kynurenine, KYNA, 3HANA and ANA could be kept in the freezer at -40ºC for at least 12 months.

To determine the stability of mixed standard solution 1, containing 200µM tryptophan, 8µM kynurenine and 100nM KYNA, aliquots of this standard solution were prepared as described in Section 3.2.4 and were stored at -40ºC for 12 months. Mixed standard solution 1 was freshly prepared from freshly prepared individual standards again as described in Section 3.2.4 and was frozen at -40ºC and used within one week. For both the stored and freshly prepared mixed standard, 6 aliquots were diluted to prepare 6 sets of 8 standards similar to those normally used for calibration. A calibration curve, required for analysing the standards as if they were samples, was prepared from one set of freshly prepared standards. A similar analysis was carried out comparing standard solutions of 2mM 3HANA and 2mM ANA, which had been stored for 12 months at -40ºC, with freshly prepared solutions, which had been frozen for up to one week. Two way analysis of variance (ANOVA) (using the General Linear Model), with a Tukey multiple comparisons post test, was used to evaluate if the stored standards were significantly different from the freshly prepared standards.

Tryptophan levels were significantly higher for solutions which had been stored for 12 months, at 7 of the 8 different concentrations (Figure 3-4A). Levels of kynurenine were also significantly higher at the highest three concentrations for the solutions which had been stored for 12 months (Figure 3-4B). The concentrations of KYNA did not change significantly on storage at -40ºC (Figure 3-4C).

The concentrations of ANA were not significantly different after storage at -40ºC for 12 months, except at the concentration of 32nM, where higher ANA was determined for the stored standards (Figure 3-5A). The two way ANOVA showed an overall decrease in 3HANA concentrations with 12 months storage. Individually this decrease was only significant at 2 of the 8 concentrations and a significant increase was shown at 32nM (Figure 3-5B).

The only metabolite to show an overall decrease on storage, which would indicate degradation, was the 3HANA standard, but this was not consistent at every concentration. From these results, standards of 3HANA were not stored for 12 months, but prepared at least every 6 months for calibration curves used in this project. The significantly higher levels of tryptophan and kynurenine measured in the frozen standards may be due to a difference in the initial stock solution of these standards. The mixed standand 1 (tryptophan, kynurenine and KYNA) and ANA standards were kept for up to 12 months for use in the calibration curves in this project.

3.4.2 Stability of Plasma Samples at -40ºC

Blood samples for each study were stored frozen often for several months before analysis. Plasma extracts were also stored frozen for a few months before analysis, as it was practical to extract a large batch of samples and then later analyse them by HPLC. It was therefore important to evaluate if the kynurenines were stable in plasma stored at -40ºC for up to 12 months and if the kynurenines in plasma extracts were stable at -40ºC for up to 6 months.

Blood samples from six healthy subjects were collected into EDTA vacutainers. Blood samples were kept on ice for a few minutes and centrifuged at 3000g for 15 minutes at 4ºC. 4 aliquots of approximately 1ml of the resulting plasma were taken for each subject and were frozen at -40ºC.

Extractions (Section 3.1.2) and HPLC analyses of the first aliquot were carried out within 1 month of collecting the bloods. Further extractions of the other 3 plasma aliquots were carried out after 3, 6 and 12 months storage of the plasma at -40ºC. On each occasion, plasma extracts (in PCA) were frozen at -40ºC in 4 aliquots of 175µl and 2 aliquots were analysed within a week and the remaining 2 after either 1, 3 or 6 months. All were analysed using the HPLC method for tryptophan, kynurenine and KYNA (Section 3.2.3) and a second aliquot of extract analysed using the method for 3HANA and ANA (Section 3.3.3). Repeated measures ANOVA with a Dunnett's multiple comparisons post test was carried out for the plasma samples to evaluate if there was any change in the results on storage compared with the initial measurement. The extracts were analysed by paired t tests to examine the effect of storing the extracts for 1, 3 or 6 months. There was only enough extract from each sample to analyse at one further time point and therefore extracts after 1, 3 and 6 months were from different original extractions.

The results for the stability of plasma samples at -40ºC are shown in Figures 3-6 and 3-7. Tryptophan and kynurenine concentrations were stable in the plasma samples stored for 3 and 6 months, but were significantly higher than the original after 12 months storage. KYNA levels were stable at all time points. The ANA and 3HANA results were more variable with storage time, with higher levels of ANA after 6 months storage, which had returned to original levels after 12 months storage. The 3HANA concentrations in the plasma samples were seen to decrease at 3 months storage, increase at 6 months storage and return to the original levels after 12 months. Storing plasma samples for up to 12 months did not show any indication that these 5 kynurenines degrade over time as there were no significant reductions in their levels over the 12 month period. The increases observed with tryptophan and kynurenine after 12 months storage were relatively small. It was concluded that plasma could be stored in the freezer at -40ºC for up to 12 months.

The stability of PCA extracts are shown in Figures 3-8 and 3-9. Storing the PCA extracts at -40ºC, decreased the concentrations of tryptophan at 1 and 6 months, increased the levels of kynurenine at 3 and 6 months and increased the level of KYNA at 1 month. Levels of ANA were increased significantly at all 3 storage times and 3HANA levels were reduced at 1 and 3 months storage. Although there was not a greater change with increased length of storage time, there were several significant changes in concentrations of most of the kynurenines, suggesting that the PCA extracts should be stored at -40ºC for a minimum length of time. Alternatively it may be acceptable if all the extracts for a study were stored at -40ºC for the same length of time.

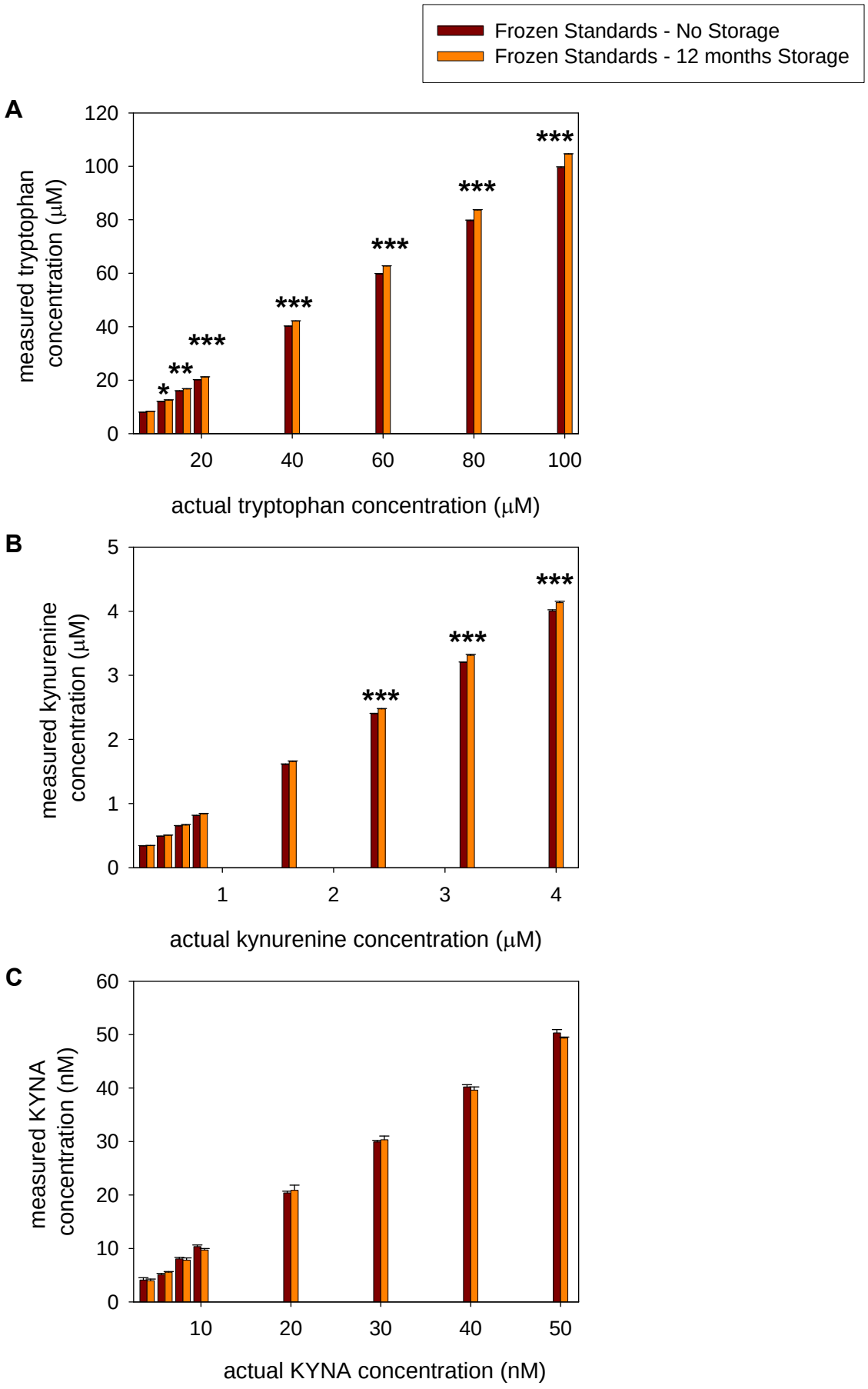


Figure 3-4 Effect of storing stock mixed standard solutions containing 200μM tryptophan, 8μM kynurenine and 100nM kynurenic acid (KYNA) for 12 months at -40ºC. Results are for standards diluted as used in calibrations. Values are mean ± SEM (n=6). Significant differences on storage: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

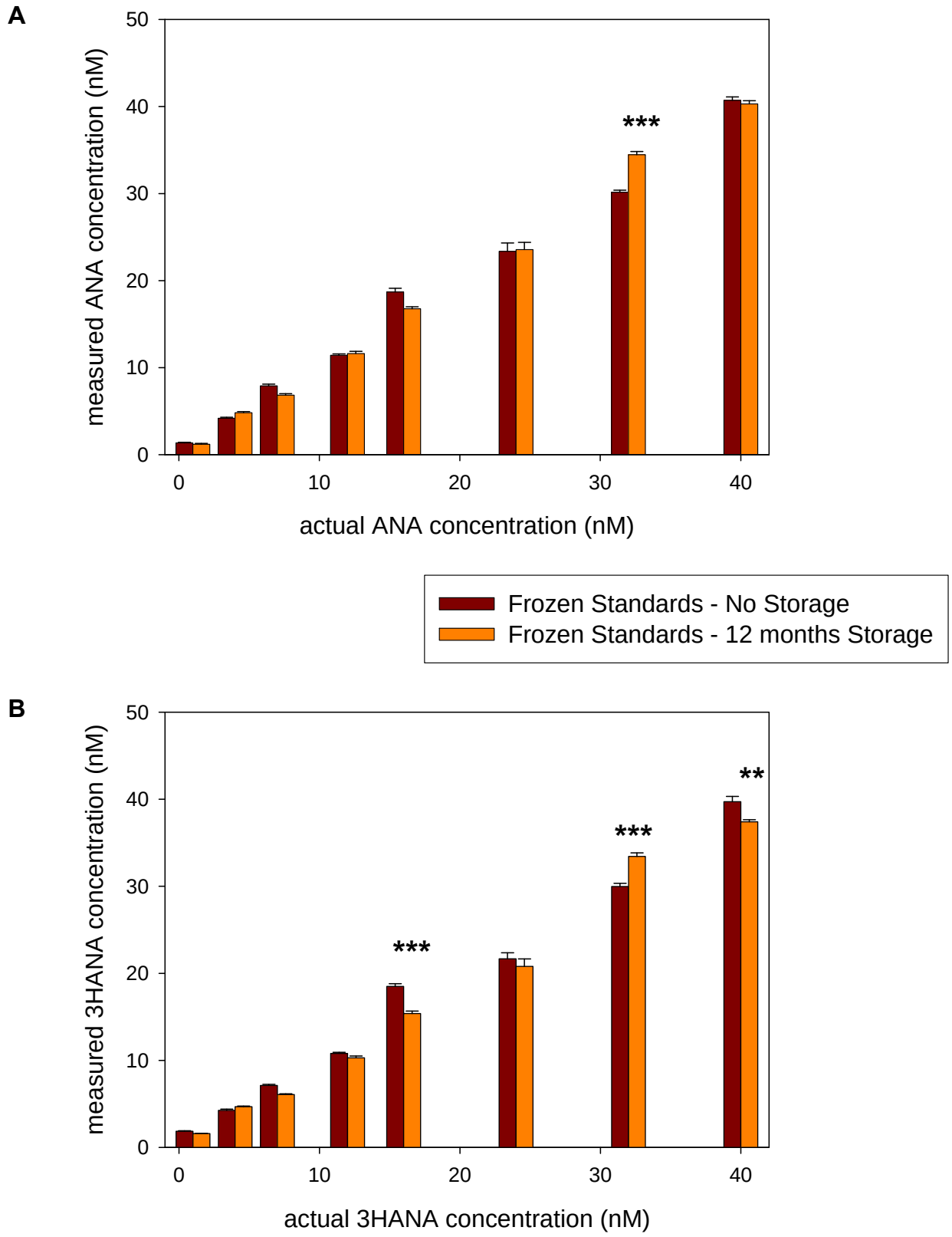


Figure 3-5 Effect of storing stock standard solutions of 2mM anthranilic acid (ANA) and 2mM 3-hydroxyanthranilic acid (3HANA) for 12 months at -40ºC. Results are for standards diluted as used in calibrations. Values are mean ± SEM (n=6). Significant differences on storage: ** $p<0.01$, *** $p<0.001$.

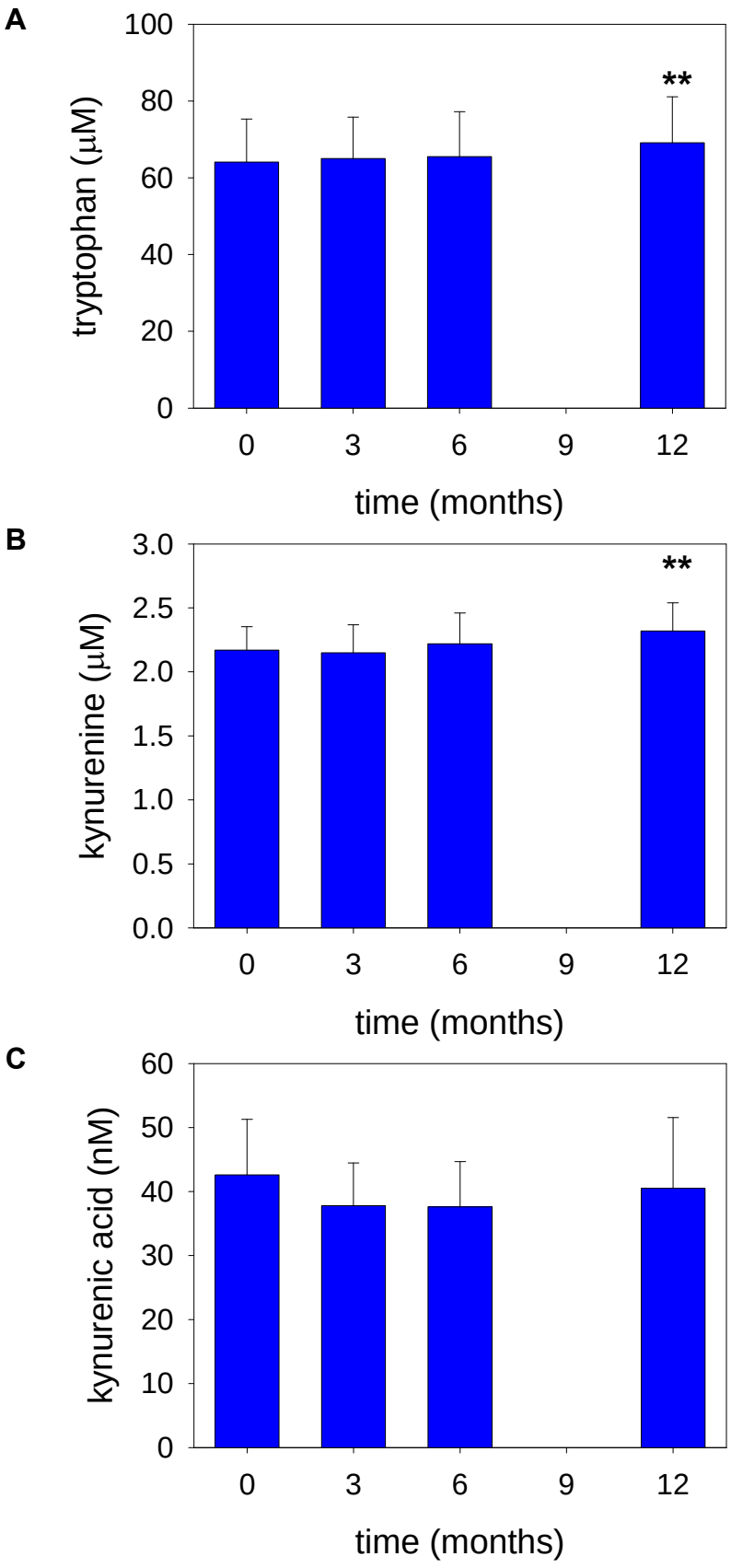


Figure 3-6 Effect of storing plasma at -40ºC for different periods of time on the levels of tryptophan (A), kynurenine (B) and kynurenic acid (C). Values are mean ± SEM (n=6). Significant differences: ** $p<0.01$.

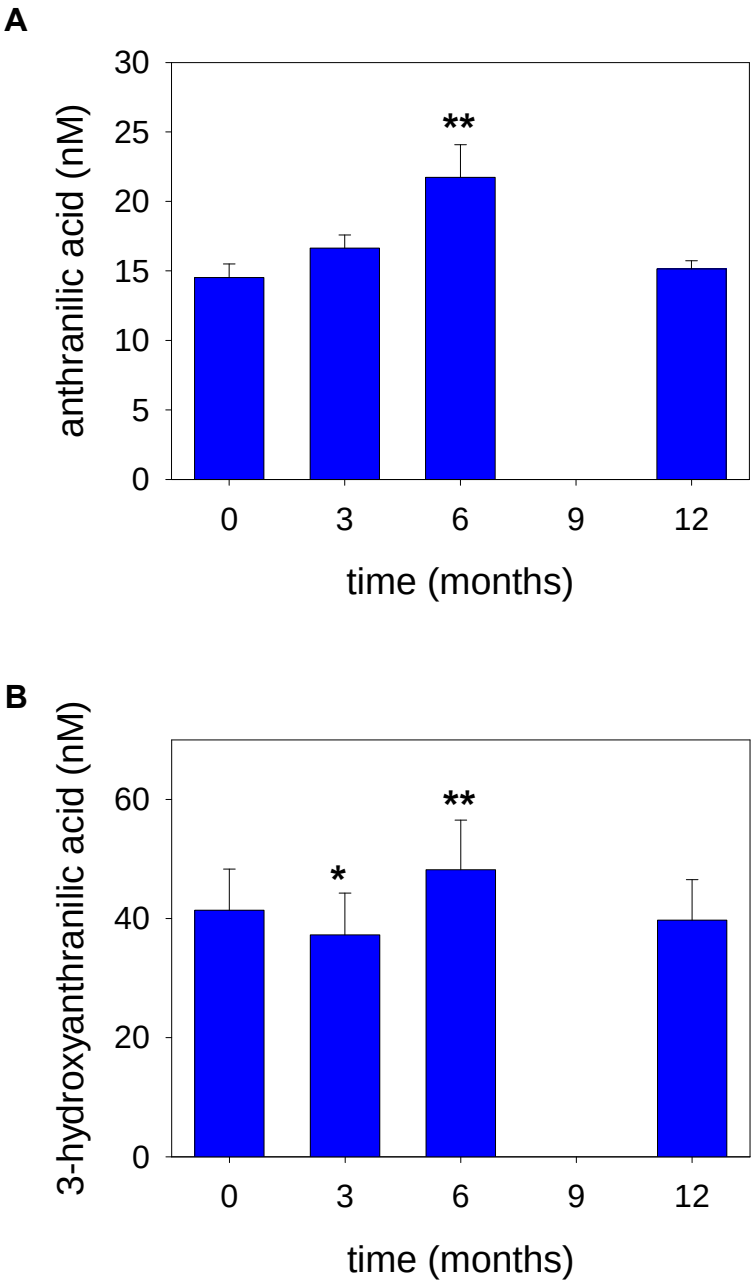


Figure 3-7 Effect of storing plasma at -40ºC for different periods of time on the levels of anthranilic acid (A) and 3-hydroxyanthranilic acid (B). Values are mean ± SEM (n=6). Significant differences: * $p<0.05$, ** $p<0.01$.

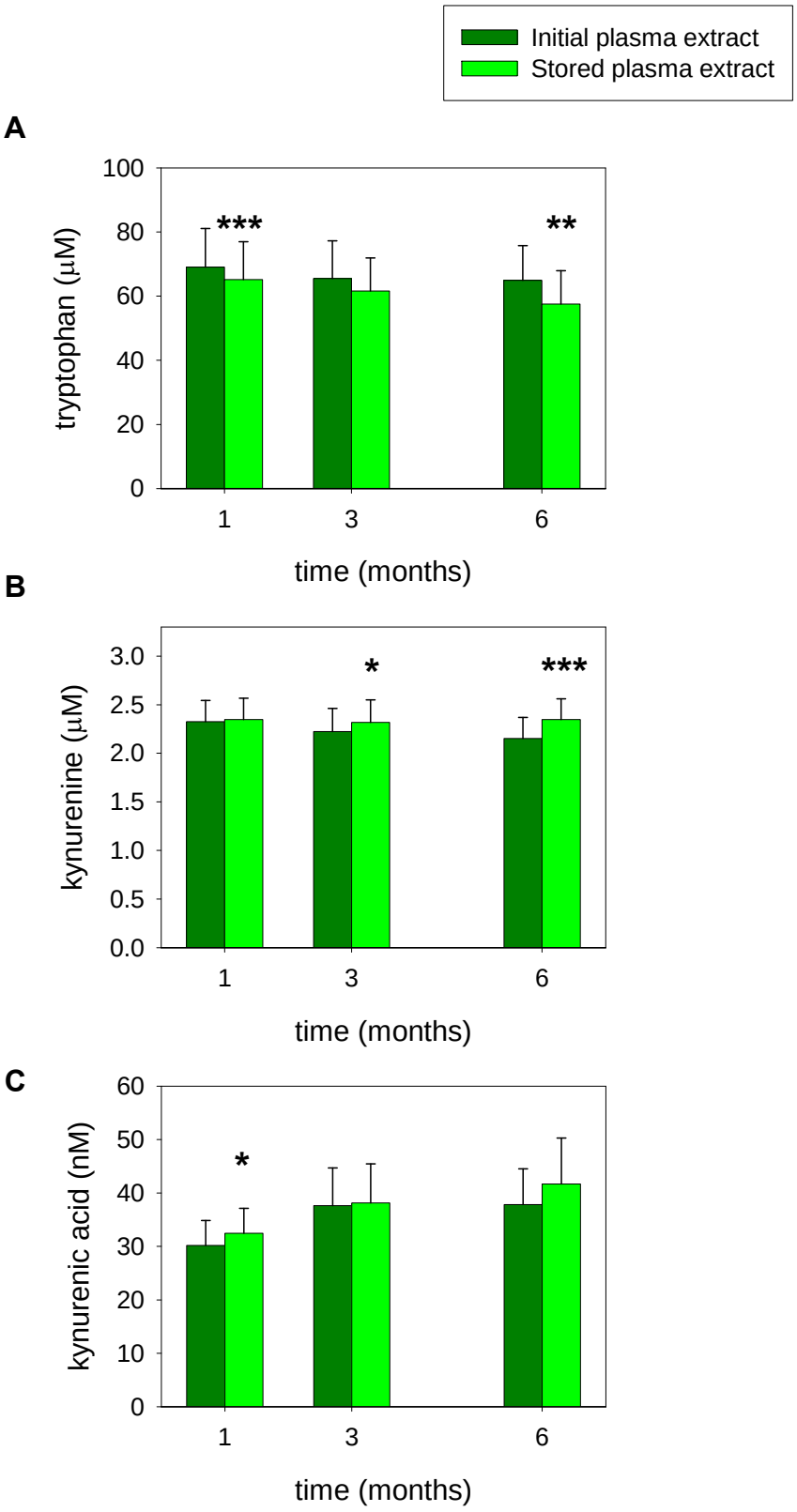


Figure 3-8 Effect of storing perchloric acid extracts of plasma at -40ºC for different periods of time on the levels of tryptophan (A), kynurenine (B) and kynurenic acid (C). Values are mean ± SEM (n=6). Significant differences: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

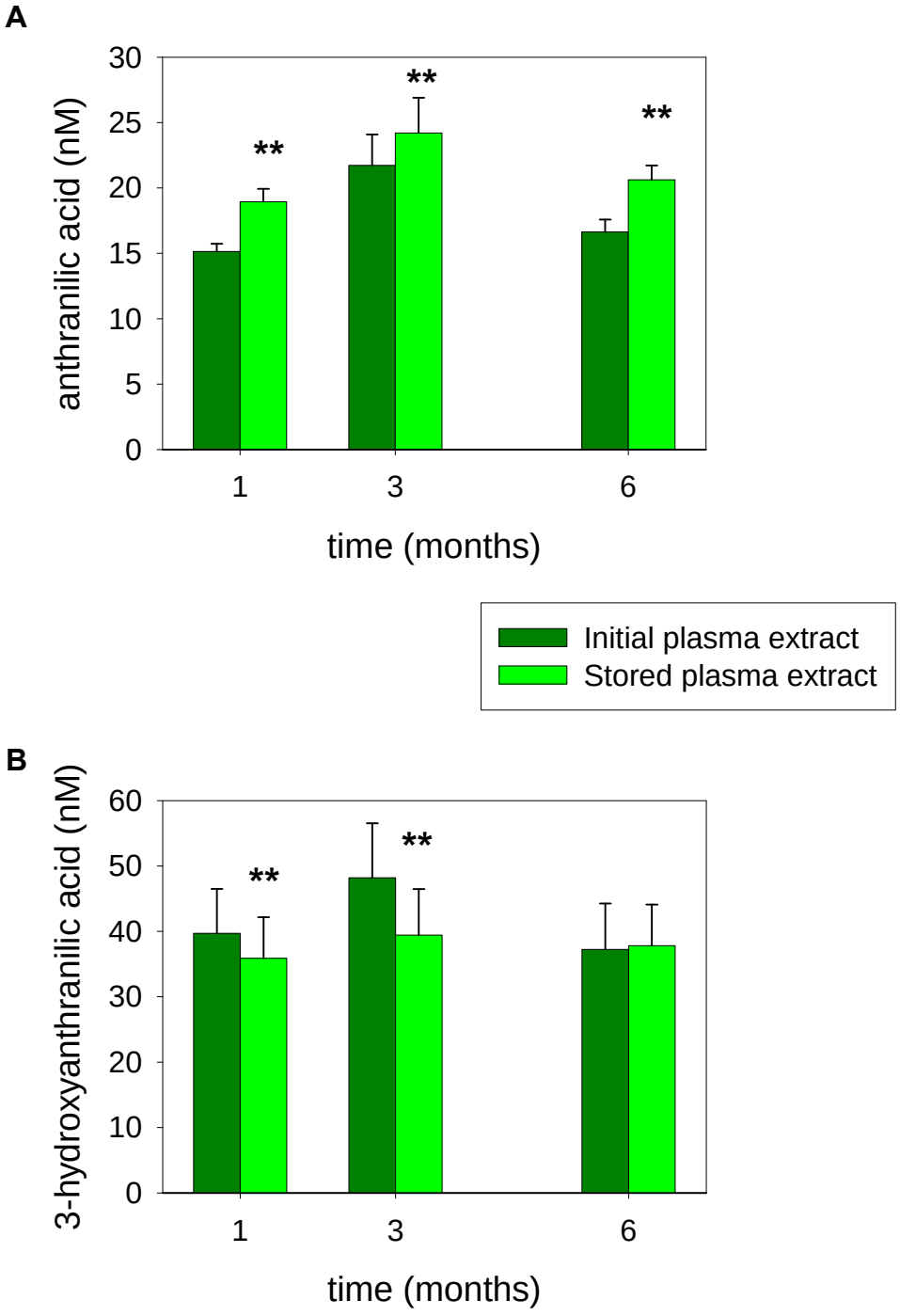


Figure 3-9 Effect of storing perchloric acid extracts of plasma at -40ºC for different periods of time on the levels of anthranilic acid (A) and 3-hydroxyanthranilic acid (B). Values are mean ± SEM (n=6). Significant differences: ** $p<0.01$.

3.5 Measurement of 3HKYN and 3HANA using electrochemical detection

3.5.1 Determination of 3HKYN and 3HANA Introduction

The levels of 3HKYN in plasma were often lower than 100nM, which was the LoD with absorbance (Section 3.2.2) and hence a more sensitive method was sought. The aim was to set up a method to measure the low concentrations of 3HKYN in plasma using HPLC with electrochemical detection, based on the methods described by Heyes and Quearry (1988) and Pearson and Reynolds (1991), where 3HKYN levels of less than 10nM could be detected. Using their method, Pearson and Reynolds (1991) were also able to quantify 3HANA in cortical tissue, although in plasma, there were other peaks interfering with the 3HANA peak. 3HKYN and 3HANA are both susceptible to oxidation when a low potential is applied, and therefore potentially could both be measured using electrochemical detection in a single analysis.

3.5.2 Method Development for 3HKYN and 3HANA

A Synergi Hydro HPLC column was used, as this column contains a C18 bonded phase endcapped with a polar group, which increases the retention of polar compounds compared to the standard C18 Kingsorb column, and can be used with completely aqueous mobile phases. One of the problems with measuring 3HKYN using the method described in Section 3.2.3 was that 3HKYN is extremely polar in comparison with the other metabolites of interest and was eluted early in the chromatogram at a similar retention time as many other compounds.

Adjusting the mobile phase to increase the retention time for 3HKYN to allow its separation from interfering components, would also result in increasing the retention times for all the other detectable compounds in plasma and the analysis time would be extremely long. A mobile phase containing the ion pair reagent, heptane sulphonic acid, was examined. The carbon chain of the heptane sulphonic acid, associates with the C18 chains of the stationary phase of the column, and allows the SO_3^- ion to be free to form an ionic bond with the NH_3^+ ion of weak base analytes, such as 3HKYN, which are ionised at a low pH. Using the ion pair reagent should retain 3HKYN on the column for a longer time and should avoid the need for long analysis times to elute all the other components of plasma

which are also electrochemically active at the oxidation voltage used. This ion pair reagent should not affect the retention of 3HANA.

A mobile phase recommended by Waters for electrochemical detection was examined, comprising 50mM phosphoric acid, 50mM citric acid, 60µM sodium EDTA, 0.5mM heptane sulphonic acid and 2mM sodium chloride, with the pH adjusted to 3.1 with 5M potassium hydroxide and the addition of 5% methanol. This mobile phase resulted in plasma samples showing interference with the 3HKYN peak.

As heptane sulphonic acid is present at a low concentration (0.5mM) in this mobile phase, increasing the concentration of the ion pair reagent to several higher concentrations was examined to increase the retention time for 3HKYN in an attempt to separate 3HKYN from the interfering compounds in plasma. The retention time for 3HKYN was increased from 15.5 minutes to 20.0 minutes by increasing the concentration of heptane sulphonic acid from 0.5mM to 8mM. The levels of methanol in the mobile phase were also adjusted to achieve the best separation of 3HKYN. The optimised mobile phase for 3HKYN and 3HANA was with 8mM heptane sulphonic acid and 5% methanol, which resulted in retention times of 20.0 minutes for 3HKYN and 25.0 minutes for 3HANA.

Peak oxidation voltage for 3HKYN is +0.65-0.70V but Heyes and Quearry (1988) used +0.55 to +0.60V, and Pearson and Reynolds (1991) used +0.60V, to ensure minimal interference in the brain samples being analysed. For 3HANA, Christen and Stocker (1992) used an oxidation voltage of +0.50V. As the oxidation voltage was decreased from +0.65V to +0.60V and +0.55V, the sensitivity for 3HKYN and 3HANA decreased, with no benefit of reduced interference. Hence, the oxidation voltage of +0.65V was used.

This mobile phase was used for the HD and chronic brain injury studies but was not ideal, as the LoD was high for both metabolites. This lack of sensitivity was due to the mobile phase having a high background current of approximately 6nA. Even with the ion pair reagent in the mobile phase, an analysis time of 70 minutes was necessary to elute all the non-polar compounds in the plasma samples, otherwise large broad interfering peaks were present in chromatograms due to carry over from the previous run.

For the HD and chronic brain injury studies, 3HKYN and 3HANA could be detected in the majority of baseline plasma samples and in all the plasma samples following tryptophan loading.

3.5.3 Final Method for the Determination of 3HKYN and 3HANA

3HKYN and 3HANA were determined using a Synergi Hydro column and electrochemical detection at the oxidation voltage of +0.65V. The reverse phase HPLC was run isocratically at 37ºC, and the electrochemical detection was also performed at 37ºC. The mobile phase was 50mM phosphoric acid, 50mM citric acid, 60µM EDTA, 8mM heptane sulphonic acid and 2mM sodium chloride, with the pH adjusted to 3.1 with 5M potassium hydroxide and the addition of 5% methanol. A flow rate of 1ml/min was used.

A volume of 150µl, 50µl or 10µl of filtered sample extract or filtered standard was injected. Standard solutions were also analysed after every 10 sample runs to check for any drift in the retention times of the metabolites. Calibration curves were analysed with the samples at least twice weekly when running continuously. A new calibration curve was run after any shutdown of the HPLC. A blank, containing only water, ascorbic acid and PCA was run with each calibration curve to ensure the baseline was completely flat at the retention times for the peaks of interest.

An example chromatogram is shown in Figure 3-10.

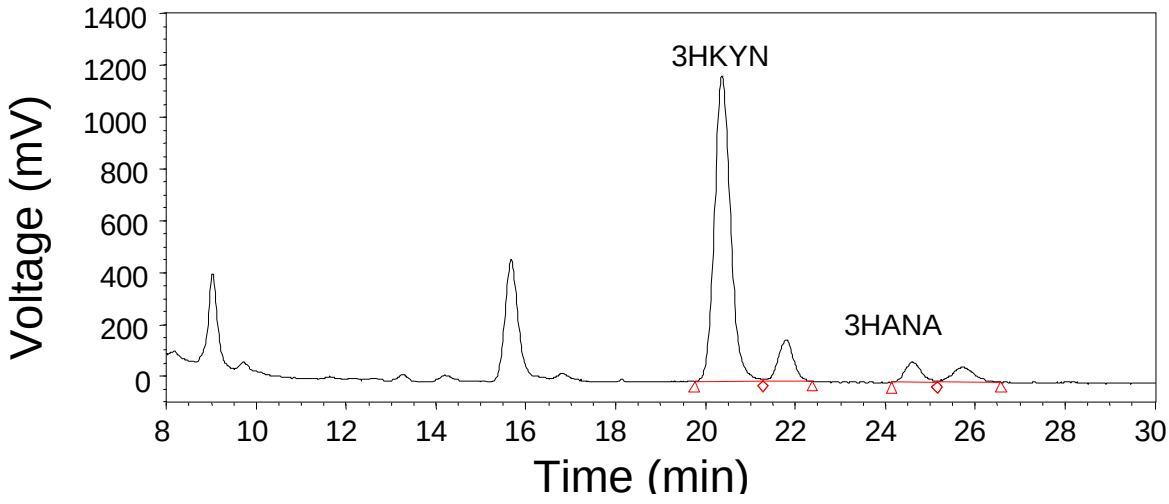


Figure 3-10 Example chromatogram of an extracted plasma sample from a patient after tryptophan loading, showing peaks for 3-hydroxykynurenine and 3-hydroxyanthranilic acid by electrochemical detection at +0.65V.

3.5.4 Preparation of 3HKYN and 3HANA Standards

2.4mM 3HKYN solution was prepared by dissolving the pure powder in HPLC grade water. To enable solubilisation, 2.4mM 3HANA was prepared by dissolving in 40mM hydrochloric acid. These stock solutions were frozen in aliquots of 1ml at -40°C.

3.5.5 Calibration Curves for 3HKYN and 3HANA

The frozen stock solutions of standards of 3HKYN and 3HANA were defrosted and mixed together using serial dilutions of up to 1 in 20. Mixed standards of equal concentration for both metabolites of 24µM, 4.8µM, 2.4µM and 0.24µM were prepared. Various volumes of these standards were diluted with water, ascorbic acid and PCA, so that they are comparable with the plasma samples. In the HD and chronic brain injury studies, as the concentrations of 3HKYN varied greatly between baseline samples and tryptophan loaded samples, 3 calibration curves were prepared using different injection volumes (150µl, 50µl and 10µl). 3HKYN could only just be detected with an injection volume of 150µl for baseline samples, whereas some loaded samples required an injection volume of 10µl. For one sample, an injection volume of 10µl was still off-scale for 3HKYN, and a dilution of 1 in 10 was made and 10µl injected. A blank of ascorbic acid and PCA was also prepared. The standards and the blank were filtered through Anopore or Alltech filter tubes using centrifugation at 10,000g for 10 minutes at 4°C. Filtrates were transferred to autosampler vials and maintained in the autosampler at 4°C. The preparation of standard solutions for the calibration curves at the various injection volumes are shown in Tables 3-19, 3-20 and 3-21. The concentrations of 3HKYN and 3HANA in the final standard solutions are given in Table 3-22.

Calibration curves were prepared from chromatogram peak areas. The sample extracts were from the same extraction as those analysed for tryptophan, kynurenine and KYNA (Section 3.1.2), and the recovery factors calculated from the areas of 3-nitrotyrosine for each extract (Section 3.2.5) were used for these electrochemical measurements. The results for the plasma extracts from the calibration curves were multiplied by 2 to give the concentration in the original plasma sample and then multiplied by the recovery factor for that sample, to produce the final concentration of 3HKYN and 3HANA in the plasma samples.

Table 3-19 Preparation of standard solutions for calibrations curves of 3-hydroxykynurenine and 3-hydroxyanthranilic acid, using a 150µl injection volume, for the Huntington's disease and chronic brain injury studies.

Standard Code	Conc of each Std in Stock Solution (µM)	Volume Mixed Std (µl)	Volume Water (µl)	Volume 2mM Ascorbic Acid (µl)	Volume PCA (µl)
Std1	0.24	100	510	40	150
Std2	0.24	200	410	40	150
Std3	0.24	300	310	40	150
Std4	0.24	400	210	40	150
Std5	0.24	500	110	40	150
Std6	2.4	100	510	40	150
Std7	2.4	150	460	40	150
Std8	2.4	170	440	40	150
Std9	2.4	200	410	40	150
Std10	2.4	250	360	40	150
BLANK		0	610	40	150

Standard (Std), 4M perchloric acid (PCA)

Table 3-20 Preparation of standard solutions for calibrations curves of 3-hydroxykynurenine and 3-hydroxyanthranilic acid, using a 50µl injection volume, for the Huntington's disease and chronic brain injury studies.

Standard Code	Conc of each Std in Stock Solution (µM)	Volume Mixed Std (µl)	Volume Water (µl)	Volume 2mM Ascorbic Acid (µl)	Volume PCA (µl)
Std1	0.24	500	110	40	150
Std2	2.4	75	535	40	150
Std3	2.4	100	510	40	150
Std4	2.4	150	460	40	150
Std5	2.4	200	410	40	150
Std6	2.4	300	310	40	150
Std7	2.4	400	210	40	150
Std8	2.4	500	110	40	150
Std9	4.8	300	310	40	150
Std10	4.8	350	260	40	150
BLANK		0	610	40	150

Table 3-21 Preparation of standard solutions for calibrations curves of 3-hydroxykynurenine and 3-hydroxyanthranilic acid, using a 10µl injection volume, for the Huntington's disease and chronic brain injury studies.

Standard Code	Conc of each Std in Stock Solution (µM)	Volume Mixed Std (µl)	Volume Water (µl)	Volume 2mM Ascorbic Acid (µl)	Volume PCA (µl)
Std1	2.4	250	360	40	150
Std2	2.4	400	210	40	150
Std3	2.4	500	110	40	150
Std4	4.8	400	210	40	150
Std5	24	100	510	40	150
Std6	24	150	460	40	150
Std7	24	200	410	40	150
Std8	24	300	310	40	150
Std9	24	350	260	40	150
Std10	24	500	110	40	150
BLANK		0	610	40	150

Table 3-22 Concentrations of 3-hydroxykynurenine and 3-hydroxyanthranilic acid in standards for calibration curves for the Huntington's disease and chronic brain injury studies.

	Conc of each standard (µM)		
	150µl injection	**50µl injection**	**10µl injection**
Std1	0.03	0.15	1.2
Std2	0.06	0.225	1.5
Std3	0.09	0.3	2.4
Std4	0.12	0.45	3
Std5	0.15	0.6	4.5
Std6	0.3	0.9	6
Std7	0.45	1.2	7.5
Std8	0.51	1.5	9
Std9	0.6	1.8	10.5
Std10	0.75	2.1	15

3.5.6 Method Validation for the HPLC Method for 3HKYN and 3HANA

3.5.6.1 Linearity of HPLC Response for 3HKYN and 3HANA

3HKYN and 3HANA standards showed a linear response from the electrochemical detector with increasing concentrations. This was observed across the range of concentrations in the calibration curves from 30nM to 750nM when an injection volume of 150µl was used, from 0.15µM to 2.1µM when an injection volume of 50µl was used and from 1.2µM to 15µM when an injection volume of 10µl was used, for both 3HKYN and 3HANA.

3.5.6.2 LoD for 3HKYN and 3HANA

Standard solutions of 3HKYN and 3HANA were diluted to determine the LoD at a signal to noise ratio of 3, using the highest injection volume for this method of 150µl. The LoD was 10nM for 3HKYN and 3HANA standards, which equates to 1.5pmoles on column.

Tryptophan depleted samples from the HD and chronic brain injury studies were examined to determine the LoD for plasma samples. The LoD with plasma samples was 40nM for 3HKYN and 40nM for 3HANA. These are the levels in the original plasma, having accounted for the 2-fold dilution of the plasma prior to analysis.

3.5.6.3 Recoveries for 3HKYN and 3HANA

Three samples of plasma were spiked with three mixed standard solutions, each containing the same concentrations of 3HKYN and 3HANA and were compared with the three original unspiked samples.

Mixed standard solutions of 3HKYN and 3HANA were prepared by defrosting and diluting stock solutions of 2mM individual standards and mixing them to give the appropriate concentrations of each standard for a 20µl spike, for each level of spike (Table 3-23). All standard solutions were prepared in 100µM ascorbic acid.

Samples were extracted using the method described for recoveries in Section 3.2.6.3, where recovery calculations are also shown. The injection volume was 50µl for standards

and samples. The concentration of plasma was determined by multiplying the result from the calibration curve by 2, and multiplying by the recovery factor.

The recoveries for 3HKYN were between 103% and 108% and for 3HANA were between 101% and 109% (Table 3-24). These results show that this electrochemical method accurately quantifies 3HKYN and 3HANA concentrations in plasma.

Table 3-23 Concentrations of a mixed standard solution containing equal concentrations of 3-hydroxykynurenine and 3-hydroxyanthranilic acid used to spike plasma to determine recoveries.

	Spike conc. for 20μl spike (μM)	**Conc. of spike if had been in plasma (μM)**
Low level	20	0.8
Medium level	40	1.6
High level	60	2.4

Table 3-24 Recoveries of 3-hydroxykynurenine and 3-hydroxyanthranilic acid using three different plasma samples spiked with three concentrations of each metabolite.

	Spike Conc as if in plasma (μM)	**Plasma 1**		**Plasma 2**		**Plasma 3**	
		Conc in plasma (μM)	**Recovery (%)**	**Conc in plasma (μM)**	**Recovery (%)**	**Conc in plasma (μM)**	**Recovery (%)**
3HKYN (μM)		0.052		0.065		0.623	
	0.4	0.483	108	0.479	104	1.045	105
	0.8	0.889	105	0.920	107	1.469	106
	1.2	1.302	104	1.302	103	1.866	104
3HANA (μM)		0.126		0.343		0.611	
	0.4	0.530	101	0.778	109	1.041	107
	0.8	0.941	102	1.211	109	1.474	108
	1.2	1.372	104	1.613	106	1.838	102

3.5.6.4 Intra-Assay and Inter-Assay Precision for 3HKYN and 3HANA

From the HD study, several plasma samples from healthy controls, 7 hours after consuming a tryptophan load, were pooled to produce a sample for repeatability (intra-assay) and a sample for reproducibility (inter-assay).

One sample was extracted 8 times by the standard procedure (Section 3.1.2), and extracts frozen at -40°C. 8 samples were analysed together in the same run, to obtain a measure of repeatability. The concentrations of 3HKYN were 3.71 ± 0.072 μM (mean ± SD), with CV = 2.0% and for 3HANA, the mean concentration was 0.186 ± 0.005 μM, CV = 2.6%.

For reproducibility, 8 aliquots of pooled plasma were extracted on the same day and frozen at -40°C. One sample was then analysed each day, together with other samples being analysed on that day, for the following 8 days. These 8 repeats were analysed using 3 different calibration curves. The concentrations for 3HKYN were 3.83 ± 0.097 μM (mean ± SD), with CV = 2.5%, and for 3HANA, the mean concentration was 0.41 ± 0.020 μM, CV=4.9%.

The CV results were all below 5% indicating good precision for this method.

3.5.6.5 Confirmation of Peak Identity for 3HKYN

To clarify that the 3HKYN peak identified by this method was indeed pure 3HKYN, 6 tryptophan loaded plasma samples were also measured using the absorbance detection at 365nm. The results are shown in Table 3-25. There was on average a 5% difference between methods, showing that the peaks were indeed 3-hydroxykynurenine. The alternative method produced some higher and some lower results than the original method as expected due to normal variability.

Table 3-25 Measurement of 3-hydroxykynurenine using an alternative method to check that the peak identified is indeed pure metabolite.

Plasma Sample	**3-Hydroxykynurenine (μM)**	
	standard method ECD +0.65V	absorbance 365nm
1	2.56	2.59
2	4.21	3.98
3	1.40	1.29
4	2.35	2.44
5	3.38	3.11
6	1.70	1.65

3.6 Measurement of 3HKYN using Solid Phase Extraction with Electrochemical Detection

3.6.1 Solid Phase Extraction for 3HKYN Measurement Introduction

A sensitive method is required to determine 3HKYN in plasma samples. The method described in Section 3.5 was not ideal, firstly, as sensitivity was low with the LoD being close to the level of 3HKYN in control plasma samples and, secondly, an HPLC run time of 70 minutes was required to elute non-polar compounds which had much longer retention times than the polar 3HKYN. Solid phase extraction (SPE) is a technique used to clean up a sample, by selectively extracting and purifying target analytes, prior to HPLC analysis. This is achieved using syringe barrels packed with a sorbent (often reverse phase or ion exchange), where some components of the sample will be absorbed on to the sorbent and others will pass through the sorbent. Different solutions of varying polarity or pH can be drawn through the cartridges at low flow rates (under vacuum using a SPE vacuum manifold) to specifically remove different compounds from the sorbent. For measuring 3HKYN in plasma, a sample clean up stage using SPE would be useful to remove the non-polar components of plasma prior to HPLC analysis, and may give the additional benefit of removing peaks which could interfere with the 3HKYN peak on the chromatogram. SPE may offer a means of concentrating the samples, either by using a low volume to elute the 3HKYN from the SPE cartridge or by using an elution solvent which could subsequently be concentrated by evaporation under nitrogen (for organic solvents) or freeze-drying (for aqueous solvents).

The mobile phase chosen for the determination of 3HKYN was based on that described by Heyes and Quearry (1988) and consisted of 0.27mM sodium EDTA, 2mM sodium chloride, 8.9mM heptane sulphonic acid, 100mM phosphoric acid, 0.9% triethylamine and 1.5% acetonitrile. This mobile phase was preferred to that used for the previous determination of 3HKYN (Section 3.5.3) as it gave a reduced background current of approximately 3nA with an oxidation voltage of +0.65V using the electrochemical detector. Isocratic reverse phase HPLC was performed using a Synergi Hydro column at 30ºC. The retention time for 3HKYN with this mobile phase was 23 minutes. Initially, electrochemical detection at an oxidation voltage of +0.65V at 30ºC was used. The LoD for 3HKYN standards using this method was 5nM.

3.6.2 Solid Phase Extraction using C18 cartridges

To separate components of different polarities, a C18 based SPE sorbent can be used with elution in a relatively polar solvent. The more polar compounds would be eluted first, while the non-polar material would remain on the cartridge. Several samples of mixed plasma were used for this work. Plasma samples (500µl) were spiked with various concentrations of 3HKYN solutions and proteins were precipitated with 400µl 0.2M PCA prior to SPE. Waters SepPak (500mg/3cc) cartridges were used, and elution of 3HKYN was with methanol.

The oxidation potential for the electrochemical detector was reduced to +0.55V from +0.65V, which omitted a peak found in some plasma samples close to the retention time for 3HKYN and flattened the baseline, without reducing the size of the 3HKYN peak. An oxidation voltage of +0.55V also reduced the number of late eluting peaks in the chromatograms.

It was found that reducing the organic content of the SPE elution solvent from 100% methanol to 10% methanol and 90% water eluted all the 3HKYN and left more non-polar material absorbed on the SPE cartridge.

Plasma could be concentrated by a factor of 2, with no losses in 3HKYN, by removing the 10% methanol in the elution solvent by drying under nitrogen and then freeze-drying the remainder. The dried extract, derived from 500µl plasma was then reconstituted in 250µl mobile phase.

The major problem with this method was very variable recoveries with both plasma samples spiked with 3HKYN and the 3HKYN standards themselves. Several attempts were made to improve recoveries. One included using different SPE cartridges all based on reverse phase sorbents from various suppliers. Waters SepPak (200mg/3ml), Waters Oasis HLB (30mg/1ml), Phenomenex Strata (2g/12ml), Phenomenex Strata-X (30mg/1ml) and Varian Focus (20mg/3ml) cartridges were assessed. This work suggested that the variable recoveries for the SepPak cartridges was that much of the 3HKYN was being lost in the water wash prior to elution in 10% methanol. The Oasis HLB cartridges appeared to be the most promising with only a small amount of 3HKYN lost in the water wash (approximately 5%), but recoveries were still variable for spiked samples, ranging from 75% to 120%. When plasma samples were analysed in triplicate before and after spiking, the recoveries were greatly improved when calculated from the mean results.

The use of an internal standard would be ideal when recoveries are variable. The internal standard used by Heyes and Quearry (1988), dihydroxybenzylamide and an isomer of 3HANA, 4-amino-3-hydroxybenzoic acid, were evaluated. Both were able to be measured by electrochemical detection using the same HPLC conditions as for 3HKYN. Neither were completed eluted in 10% methanol on SPE and hence were both unsuitable for use as an internal standard.

3.6.3 Solid Phase Extraction using Cation Exchange Cartridges

An alternative SPE method for sample clean up is to use a cation exchange SPE cartridge. 3HKYN contains two amino groups and hence at low pH, below the pKa of 3HKYN, these groups will be present in their ionised form (NH_3^+). This ionised compound will be retained on a cation exchange cartridge at low pH, but can be eluted by increasing the pH and therefore changing 3HKYN to its non-ionised form. Pearson and Reynolds (1991) reported a SPE method using cation exchange for measuring 3HKYN in brain tissue and blood plasma.

A SPE procedure similar to that of Pearson and Reynolds (1991), was attempted using Phenomenex Strata-X-C (60mg/3ml) and Varian Bond Elut SCX (500mg/3ml) cartridges. After an initial extraction of 500µl plasma with 50µl 1M PCA, samples were loaded on to the SPE sorbent at pH 2.5 and eluted at pH 7 with 0.1M sodium phosphate buffer. At least 2ml of pH 7 buffer was required to elute all the 3HKYN from the SPE cartridge. The concentration of 3HKYN in this eluate was too dilute to quantify levels normally found in plasma.

To elute all the 3HKYN in a minimum volume, smaller sorbent sizes were examined. Bond Elut SCX (50mg/1ml) cartridges were compared to mixed mode cation exchange cartridges, namely Strata-X-C (30mg/1ml), Supelco Discovery DSC-MCAX (100mg/1ml) and Waters Oasis MCX (30mg/1ml), with elution in 1ml 0.1M phosphate buffer pH 7. Very poor recoveries were obtained for all 4 cartridge types for both 3HKYN standards and plasma samples.

Another approach was to concentrate the 3HKYN eluted from the SPE in a larger volume of eluate, by freeze-drying. 3HKYN was eluted from SPE cartridges in 2ml 0.1M pH 7 buffer, which was then freeze-dried. There was a large amount of crystallised phosphate buffer present in the dried eluates, which required 1ml mobile phase to resolubilise for HPLC, which somewhat defeated the purpose of concentrating the sample.

3.6.4 Solid Phase Extraction Summary

SPE using Oasis HLB cartridges and eluting in 10% methanol was a promising method of sample preparation for measuring 3HKYN in plasma but variable recoveries resulted in the method not being used. The use of an internal standard would be ideal when recoveries are variable. However a suitable internal standard which matches the behaviour of 3HKYN through SPE was not found. Alternatively, analysing all samples in triplicate and checking each SPE run with triplicate spikes was shown to produce good recoveries but this is impractical for the large numbers of samples present in the patient studies in this project.

Cation exchange based SPE was also attempted, but the SPE eluate was too dilute to detect the 3HKYN levels present from the plasma. As this is a more specific SPE separation, it would be worth pursuing in the future, using a elution solvent of the same pH but with a lower molarity which could be concentrated by freeze-drying. This method of sample clean up, together with the improved mobile phase for electrochemical detection, has the potential as a future method for determining 3HKYN concentrations in plasma.

4 Huntington's Disease

4.1 Introduction

There is much evidence for altered kynurenine pathway metabolism in post-mortem brain tissue from HD patients, with Guidetti *et al*. (2004) demonstrating increases in the levels of QUIN and 3HKYN, with no change in the levels of KYNA, in early stage HD. Post mortem brain tissue at later stages of the disease do not appear to have increased QUIN levels, but have shown a significant increase in the ratio kynurenine: KYNA (Beal *et al.*, 1990) and increases in the levels of 3HKYN (Pearson and Reynolds, 1992). There is also evidence of oxidative stress in post-mortem HD brains (Browne *et al*., 1999).

In this study, the patients were at the late stages of HD and blood samples were analysed to assess changes in the kynurenine pathway. The dynamics of the kynurenine pathway in these living patients was examined by acutely depleting tryptophan from the diet and giving a single tryptophan load. This demonstrated how the kynurenine pathway functions under extreme lack or excess of tryptophan and may give a clearer understanding of how this pathway differs between HD patients and healthy control subjects. In addition, tryptophan depletion should reduce the production of the neurotoxic kynurenines, QUIN, 3HKYN and 3HANA, and, although tryptophan is an essential amino acid in the diet, an appropriate diet with a reduced tryptophan content may offer some benefit for HD patients (Pascoe, 1993). However potential drugs able to block the production of neurotoxic products of the kynurenine pathway may be more suitable and offer much more benefit than a low tryptophan diet. Tryptophan loading is expected to increase metabolism via the kynurenine pathway and hence increase the levels of all the kynurenines. The balance between the neuroprotective KYNA and the neurotoxic kynurenines is expected to be important for neurotoxicity (Guidetti *et al*., 2000). Tryptophan loading could lead to increased oxidative stress as more of the neurotoxic products able to produce reactive oxygen species (3HKYN, 3HANA and QUIN) are formed.

As the kynurenine pathway is the major metabolic pathway for tryptophan in most tissues after protein synthesis, oral tryptophan depletion and loading is expected to produce large changes in the levels of kynurenines. Manipulating tryptophan will also affect other tryptophan pathways including the pathway which produces 5HT. Oral tryptophan depletion and loading have been used as tools to reduce or increase 5HT in the brain to examine the role of 5HT in depression and other behavioural disorders. In addition, blood

tryptophan concentrations will affect the entry of other large neutral amino acids into the brain, as this group of amino acids are in competition for the same active transport system to cross the blood-brain barrier.

The mechanism for the tryptophan deficient amino acid mixture to deplete tryptophan from the blood was due to its induction of protein synthesis. A large decrease in tryptophan levels in both plasma and brain tissue of rats following administration of the tryptophan depleting mixture was demonstrated, which did not occur after pretreating the rats with a protein synthesis inhibitor, cycloheximide (Moja *et al*., 1991).

Our depletion protocol, involving ingestion of 100g of a tryptophan deficient amino acid mixture, after an overnight fast, has been used in several studies which have demonstrated the time for a maximum depletion in plasma tryptophan was 5 hours, falling by 80-90% in healthy males (Young *et al*., 1996). The changes associated with tryptophan depletion are transient and resolve within 24 hours (Reilly *et al*., 1997) and in our study, tryptophan loading was given at 24 hours after the depletion mixture, when kynurenine levels were expected to have returned to normal.

A normal Western diet contains about 0.5g of tryptophan daily. The single tryptophan load in our study was 6g tryptophan. Previous work has used doses of tryptophan of 3g (Joseph and Risby, 1975), 5g (Huether *et al*., 1992) or 10.3g (Bjork *et al*., 1999), again having been studied with regard to depression and behavioural disorders. Plasma tryptophan levels were shown to have returned to their original levels by 8 hours after a 3g tryptophan load (Joseph and Risby, 1975).

Tryptophan loading stimulates metabolism along the different tryptophan metabolic pathways, and also increases the rate of protein synthesis, utilising other amino acids in peripheral tissues (Huether *et al*., 1992).

Previous work on the role of kynurenines in HD has focused on post-mortem human brains and on animal models of the disease. This study examines the levels of kynurenines in the blood of living HD patients. If kynurenine levels in the blood reflect results from post-mortem brains and the hypotheses developed for HD, blood samples would provide much more readily attainable samples for further studies of this disease and may provide useful markers of the disease. As there are no adequate treatments for HD available at present, this study may indicate if modulation of the kynurenine pathway would be useful for potential therapeutic openings in HD.

Tryptophan, kynurenine, KYNA and QUIN were determined for baseline, tryptophan depleted and tryptophan loaded blood samples for each subject. 3HKYN, 3HANA, neopterin and lipid peroxidation were measured in baseline and tryptophan loaded samples. ESR and CRP were determined for the baseline samples.

4.2 Methods

4.2.1 Patients and Samples

All the patients were at an advanced stage of HD and required permanent hospitalisation in the Royal Hospital for Neuro-disability. 11 patients (3 males, 8 females; age 60 ± 1.7 (mean ± SEM)) and 15 healthy control subjects (4 males, 11 females; age 43 ± 2.2 (mean ± SEM)) completed the full experimental protocol and were included in the results of the study. Originally, 3 extra patients and 3 extra controls (all 6 were female) were included, but they either consumed less than 80% of the tryptophan depleting or loading mixtures or vomited within 2 hours of consumption, and hence were removed from the study results. None of the patients or controls were taking any drugs known to interfere with tryptophan metabolism, such as glucocorticoids. Permission for the study was obtained from the Ethical Committee of the Epsom and St Helier University Hospitals NHS Trust and from the Riverside Ethical Committees of the Royal Hospital for Neuro-disability. Patients and controls were enrolled by obtaining their informed written consent or the consent of their next of kin.

All subjects were fasted overnight before a baseline blood sample was taken. Immediately after this initial blood sample, the subjects consumed an amino acid mixture (100g) containing all the essential amino acids, except tryptophan. This was given in the form of a fruit flavoured drink. Figure 4-1 shows the time plan for the tryptophan depletion and loading experiment. Further blood samples were taken 5 and 7 hours later, at times when the blood was expected to contain the minimum levels of tryptophan (Young *et al*., 1996). Subjects then resumed a normal diet containing tryptophan for the remainder of the day but again fasted overnight. At 24 hours after the initial baseline blood sample, when tryptophan levels were expected to have returned to normal (Reilly *et al*., 1997), a second baseline blood sample was taken immediately before the tryptophan load was administered. The tryptophan load (6g) was again taken orally as a fruit flavoured solution. Further blood samples were taken after 5 and 7 hours when levels of tryptophan

were expected to be at a maximum (Heuther *et al*., 1992). All subjects were closely monitored by a physician throughout the study.

EDTA plasma was prepared using EDTA vacutainers to collect the blood which was then centrifuged at 1000g for 10 minutes. Serum samples were prepared by allowing blood samples to clot before centrifuging at 1000g for 10 minutes. All samples were protected from light and frozen within two hours of venesection. Samples were kept frozen while being transported from Epsom General Hospital to the University of Glasgow and then stored at -40ºC prior to analysis.

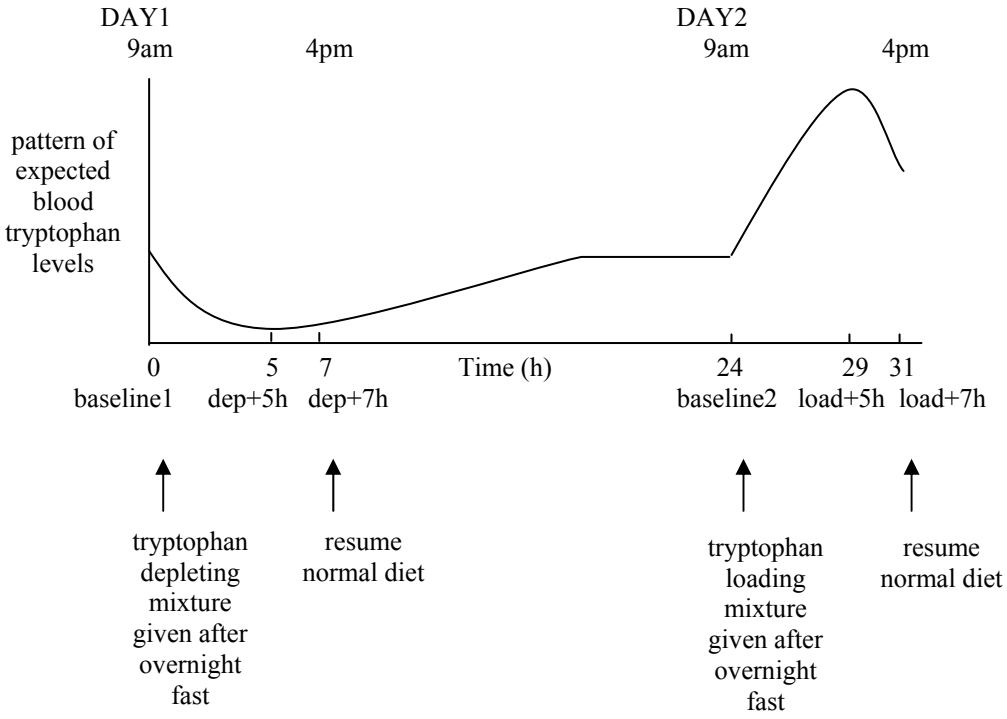


Figure 4-1 Time plan for the tryptophan depletion and loading protocols indicating time points when blood samples were taken, together with the pattern of expected changes in blood tryptophan concentrations.

4.2.2 Kynurenines by HPLC

Samples of EDTA plasma were extracted as described in Section 3.1.2. Mrs R McMillan helped with some of the extractions. Tryptophan, kynurenine and KYNA were analysed by HPLC using absorbance and fluorescence detection (Section 3.2.3). 3HKYN and 3HANA were determined by HPLC with electrochemical detection (Section 3.5.3). The levels of 3HKYN and 3HANA in tryptophan depleted samples could not be detected and therefore results were only compiled for the second baseline sample and the loaded samples.

4.2.3 Quinolinic acid by GC-MS

Samples of EDTA plasma were analysed for QUIN using GC-MS by West Park Biochemistry Laboratories at Epsom General Hospital by the method described in the appendix.

4.2.4 Neopterin and Lipid Peroxidation

Neopterin and lipid peroxidation were determined in serum by Dr C.M. Forrest at the University of Glasgow using the methods described in the appendix. Only baseline and loaded samples were analysed.

4.2.5 Clinical Analyses - ESR and CRP

ESR and CRP, standard clinical analyses, were determined for only the first baseline sample at Epsom General Hospital. The methods are briefly described in the appendix.

4.2.6 Data Analysis and Statistics

GraphPad InStat statistics package was used for all the statistical analysis, except for the correlations where NCSS97 software was used.

Comparisons between patients and controls were made using unpaired two-tailed t tests. A Welch correction or a non-parametric Mann-Whitney test was used if necessary.

A repeated measures ANOVA, followed by a Student-Newman-Keuls multiple comparison post test, was used when comparing measurements from the same patients or control

subjects at different time points during the depletion or loading protocols. Depletion samples were compared with baseline 1, with a separate ANOVA for baseline 2 and loaded samples. Two separate ANOVAs were required as the extremely large differences between depleted levels and loaded levels may mask significant differences between baseline and depleted samples. Comparison of original baseline and baseline 24 hours after depletion for the same patients or control subjects was determined using a paired two-tailed t test.

In addition to statistically analysing the levels of the kynurenines measured in the bloods of patients with HD and healthy controls, the ratios between different kynurenines were also calculated. These ratios demonstrate how much metabolite was produced from its substrate, which gives an indication of the activity of the enzyme catalysing the reaction. Differences between patients and controls in the ratios of QUIN: KYNA and 3HKYN: KYNA also show if the pathway has changed to favour one route more than another. In addition, the ratios are advantageous in that they indicate pathway activity, rather than actual levels of metabolites which can be quite variable in human subjects. Ratios were analysed using the same statistical tests as the metabolites themselves.

Another means of data analysis, primarily to overcome any masking of significant differences due to the natural variability between human subjects, was to calculate, by subtraction, the change in the levels of kynurenines from baseline to depleted or from baseline to loaded for individual subjects. This demonstrates differences between patients and controls due to the effect of the same depleting and loading mixture given to each subject, independent of the initial baseline levels. The mean change for control subjects and patients with HD have then been compared at each time point using a two-tailed t test.

In all cases a significance threshold of 5% ($P<0.05$) was employed.

Correlations between the kynurenines, the ratios of the kynurenines, neopterin, lipid peroxidation, ESR and CRP were assessed using a correlation matrix and the statistics package NCSS97. Spearman correlations were made as some of the data were non-parametric.

4.3 Results

4.3.1 Kynurenines

4.3.1.1 Tryptophan

Baseline levels of tryptophan were not significantly different between patients and controls. The only significant difference between tryptophan levels in patients and controls was at 5h after depletion, where there was more tryptophan in patients' blood compared with controls (Figure 4-2A). The loaded samples showed no significant differences between patients and controls at either 5 or 7 hours after loading (Figure 4-2B).

The depletion mixture, as expected reduced the levels of tryptophan in the bloods of both patients and controls (Figure 4-2A). This reduction in tryptophan was highly significant at both 5 and 7 hours after the subjects were given the tryptophan depleting mixture, with tryptophan levels remaining similar (not significantly different) at 5 and 7 hours. The tryptophan load produced significantly higher levels of tryptophan in the bloods of both patients and controls at both 5 and 7 hours after loading (Figure 4-2B). The levels at 7 hours were beginning to return to normal, being significantly lower at 7 hours compared with 5 hours after loading for both patients ($p<0.001$) and controls ($p<0.001$). At the second baseline, 24 hours after depletion, tryptophan levels had returned to levels similar to that of baseline 1 for both patients and controls (Figure 4-2A).

When the changes in tryptophan levels between baseline and depleted samples were calculated for each subject and the means compared, the reduction in tryptophan on depletion was significantly less in patients compared with controls at both 5 and 7 hours after depletion (Figure 4-3A). There were no significant differences between patients and controls when the increases in tryptophan on loading were assessed (Figure 4-3B).

4.3.1.2 Kynurenine

Kynurenine levels were significantly higher in patients than controls at baselines 1 and 2, at both 5 and 7 hours after depletion (Figure 4-4A) and at 5 hours after loading (Figure 4-4B). This trend was still seen 7 hours after loading, although it was not significant.

As with tryptophan, the levels of kynurenine were significantly lower at both 5 and 7 hours after depletion compared with baseline levels for patients and controls (Figure 4-4A).

There was also a significantly lower level of kynurenine at 7 hours after depletion compared with 5 hours, only in the patient group ($p<0.05$), suggesting that on depletion, kynurenine levels in the patients may take longer to reach a minimum level than in the controls. In control subjects, the initial baseline was not significantly different from baseline 2, 24 hours after depletion, but for patients, the second baseline was significantly higher than the first. For patients the effect of this tryptophan depletion may actually stimulate the pathway activity once a normal diet has been resumed.

After tryptophan loading, levels of kynurenine were significantly higher than baseline for both patients and controls (Figure 4-4B). Both patients ($p<0.001$) and controls ($p<0.01$) showed significant decreases in kynurenine levels between 5 and 7 hours after loading.

The changes between baseline levels and depleted or loaded levels were calculated and are shown in Figure 4-5, with the decrease in kynurenine concentrations being significantly greater for patients compared with controls at 7 hours after depletion. This is due to the much higher level of kynurenine at baseline. None of the other changes in kynurenine levels due to depletion or loading resulted in a significant difference between patients and controls.

4.3.1.3 Kynurenine: Tryptophan ratio

The ratio of kynurenine: tryptophan (K:T) is recognised as an indicator of IDO/TDO activity, and was calculated for all results of this study. HD patients had significantly higher K:T ratios than controls at both the first and second baseline samples and also at 5 hours after depletion (Figure 4-6A). There was no difference between the K:T ratios of patients and controls at 7 hours after depletion and at 5 and 7 hours after loading (Figure 4-6).

There seemed to be a different effect between patients and controls after depletion, with controls at 5 hours having a significantly lower K:T ratio, but patients having a significantly higher K:T ratio, compared with their respective baseline samples (Figure 4-6A). Similar K:T ratios were observed at 7 hours after depletion. As with the original kynurenine levels, the K:T ratio for patients was significantly higher at the second baseline compared with the initial baseline, suggesting increased pathway activation following the depletion protocol. On loading, the K:T ratios were significantly increased in both patients and controls, with levels being similar after 5 and after 7 hours (Figure 4-6B).

4.3.1.4 Kynurenic acid

There were no significant differences between KYNA concentrations in patients and controls at any of the time points (Figure 4-7). Tryptophan depletion caused a significant reduction in KYNA levels for both patients and controls at both 5 and 7 hours after depletion, with KYNA levels having returned to baseline values by 24 hours after depletion (baseline 2). Similarly, tryptophan loading resulted in a significant increase in the levels of KYNA at 5 hours after loading, which had significantly reduced by 7 hours after loading for both patients ($p<0.001$) and controls ($p<0.001$).

When the change in KYNA levels due to depletion or loading were calculated, there were no significant differences between patients and controls (Tables 4-1 and 4-2). There seems to be a trend that there is more of an increase in KYNA concentrations in patients after loading than in controls (Table 4-2), similar to the trend in the raw KYNA results (Figure 4-7B).

4.3.1.5 Kynurenic acid: Kynurenine ratio

The ratio of KYNA with its substrate, kynurenine (KYNA: KYN) was calculated for each subject at each time point, to indicate the activity of the KAT enzymes. At both baselines, the KYNA: KYN ratios were significantly lower for patients compared with controls (Figure 4-8A). A lower ratio was also observed for patients 7 hours after the tryptophan depletion. The kynurenine levels in many of the controls at 5 hours after depletion were undetectable and determined as zero kynurenine, and hence a ratio could not be calculated. This resulted in too few ratios to calculate a valid KYNA: KYN ratio for this time point, and therefore no data are shown on the graph for 5 hours after depletion in the controls. There were no differences between patients and controls after loading (Figure 4-8B).

The ratio was significantly lower for controls at 7 hours after depletion compared with baseline 1, but depletion did not reduce the ratios in the patients' samples. On loading, the KYNA: KYN ratio was significantly increased after 5 hours for both patients and controls and remained increased for the patients 7 hours after loading. There was a significant decrease in the KYNA: KYN ratio between 5 and 7 hours after loading for both patients ($p<0.001$) and controls ($p<0.001$).

4.3.1.6 3-Hydroxykynurenine

The levels of 3HKYN in all the depleted samples were below the LoD of the method and therefore only baseline 2 and the loaded samples were considered. The levels of 3HKYN were significantly lower in HD patients compared with controls at baseline (Figure 4-9A), although there were no significant differences after loading. The levels of 3HKYN were significantly higher after loading in the patients' samples at both 5 and 7 hours and in the control samples at 5 hours after loading. There was also a significant decrease in the levels of 3HKYN between 5 and 7 hours after loading for patients ($p<0.01$) and for controls ($p<0.05$). When the change in the levels of 3HKYN were calculated, there were no significant differences between patients and controls at either 5 or 7 hours after loading (Table 4-2).

4.3.1.7 3-Hydroxyanthranilic acid

The levels of 3HANA in all the depleted samples were lower than the LoD of the method and therefore only the second baseline and the loaded samples were assessed. At baseline 2 and at both 5 and 7 hours after tryptophan loading, levels of 3HANA were significantly lower in patients compared with controls (Figure 4-9B). For both patients and controls, the loaded samples at 5 and 7 hours were significantly higher than baseline samples, with levels significantly lower after 7 hours than after 5 hours (patients $p<0.05$, controls $p<0.01$). When the change in 3HANA levels between baseline and loading were calculated, no significant differences were observed between patients and controls, although the change in the patient group appeared to be slightly less than for the controls (Table 4-2).

4.3.1.8 3-Hydroxykynurenine: Kynurenine ratio

The ratios of 3HKYN: kynurenine (3HKYN: KYN) appeared lower for patients compared with controls (Figure 4-10A), being significant at 5 hours after loading.

4.3.1.9 3-Hydroxykynurenine: Kynurenic acid ratio

As in the case of 3HKYN: KYN, the ratio of 3HKYN: KYNA was significantly lower in patients at 5 hours after loading than in controls (Figure 4-10B).

4.3.1.10 3-Hydroxyanthranilic acid: 3-Hydroxykynurenine ratio

There were no significant differences between the ratio of 3HANA: 3HKYN for patients and controls, at baseline or at 5 or 7 hours after loading.

4.3.1.11 Quinolinic acid

There was a large variability in the QUIN results for both controls and HD patients, which made it difficult to interpret the results statistically. In both the HD patients and the controls at baseline, there was approximately a 1000-fold difference between subjects' QUIN concentrations. There was no known reason to expect any subject to have a high level of QUIN in their blood.

The only significant difference between patients and controls for QUIN was at 7 hours after depletion, when the patients had a lower level than the controls (Figure 4-11A). Tryptophan depletion did not result in a significant decrease in QUIN levels after either 5 or 7 hours. Although not significant, it appeared that QUIN levels were lower at 24 hours after depletion (baseline 2) than at the initial baseline, suggesting that the effect of the depleting mixture may still be affecting the subjects' QUIN concentrations. There was a significant increase in QUIN at 5 hours after loading for both patients and controls, which appeared to remain similar at 7 hours for the controls, but may still be increasing at 7 hours for the patients (Figure 4-11B). However, this difference between 5 and 7 hours after loading for the patients was not significant.

To attempt to overcome the large variability in QUIN levels, the change between baseline and depleted or loaded samples were examined. However the changes also produced no significant differences between patients and controls, except that patients showed a significantly lower decrease in QUIN at 7 hours after depletion (Figure 4-12), similar to the result with the raw data. The changes also suggest that the increase in QUIN concentration at 7 hours after loading was higher in patients than in controls but again was not significant.

4.3.1.12 Quinolinic acid: 3-Hydroxyanthranilic acid ratio

There were no significant differences between the ratios of QUIN: 3HANA between patients and controls, although the mean ratio was higher for the patients at all 3 time points (Figure 4-13).

4.3.1.13 Quinolinic acid: Kynurenic acid ratio

There were no significant differences in the QUIN: KYNA ratios between patients and controls at any of the time points during depletion and loading, but it was noted that the initial QUIN: KYNA ratio was higher in patients than controls at the initial baseline and that on loading, the ratios were lower for patients than controls (Figure 4-14). The ratios reflect the delayed response of QUIN compared with KYNA following depletion and loading.

4.3.2 Neopterin

Neopterin levels were significantly higher in patients compared with controls at baseline (Figure 4-15A). On loading, neopterin levels in the controls were significantly increased at both 5 and 7 hours after loading. Neopterin levels had decreased in patients at 7 hours after loading compared to baseline.

4.3.3 Lipid Peroxidation

The levels of lipid peroxidation for patients were significantly higher at baseline and at 5 and 7 hours after loading compared with controls (Figure 4-15B). As with neopterin, the level of lipid peroxidation in controls increased on loading, being significantly higher than at baseline at both 5 and 7 hours after loading. There was little change in lipid peroxidation in the patients on loading.

4.3.4 ESR and CRP

Baseline ESR levels were not significantly different between HD patients and controls (Figure 4-16A). The baseline CRP level in patients was significantly higher than in controls (Figure 4-16B).

4.3.5 Correlations

As the number of HD patients was only 11, and control subjects only 15, fewer significant correlations than would be observed with a larger group were expected. However significant correlations were observed between some of the kynurenines in HD patients and in controls. The more meaningful or interesting significant correlations are described.

For HD patients, there was a significant positive correlation between neopterin and the K:T ratio ($p<0.05$) at baseline. Neopterin also correlated negatively with the KYNA: KYN ratio ($p<0.05$) at baseline, demonstrating that although inflammation increased the K:T ratio, it may inhibit the formation of KYNA from kynurenine. There were also significant negative correlations after depletion between the K:T ratio and KYNA levels ($p<0.05$ at 5 hours after depletion, $p<0.05$ at 7 hours after depletion). In contrast, controls at the first baseline showed a positive correlation between K:T ratio and KYNA ($p<0.05$).

Controls showed positive correlations between K:T ratio and 3HANA at both 5 and 7 hours after loading ($p<0.01$, $p<0.05$ respectively), not observed for the patients.

In controls, there was a positive correlation between age and neopterin at 5 hours after loading ($p<0.05$), but no similar correlations for the HD patients. Interestingly there were positive correlations between age and KYNA levels, for controls at both baselines ($p<0.05$ at baseline 1 and $p<0.05$ at baseline 2) and for HD patients at the second baseline ($p<0.05$) and at 5 hours after depletion ($p<0.05$).

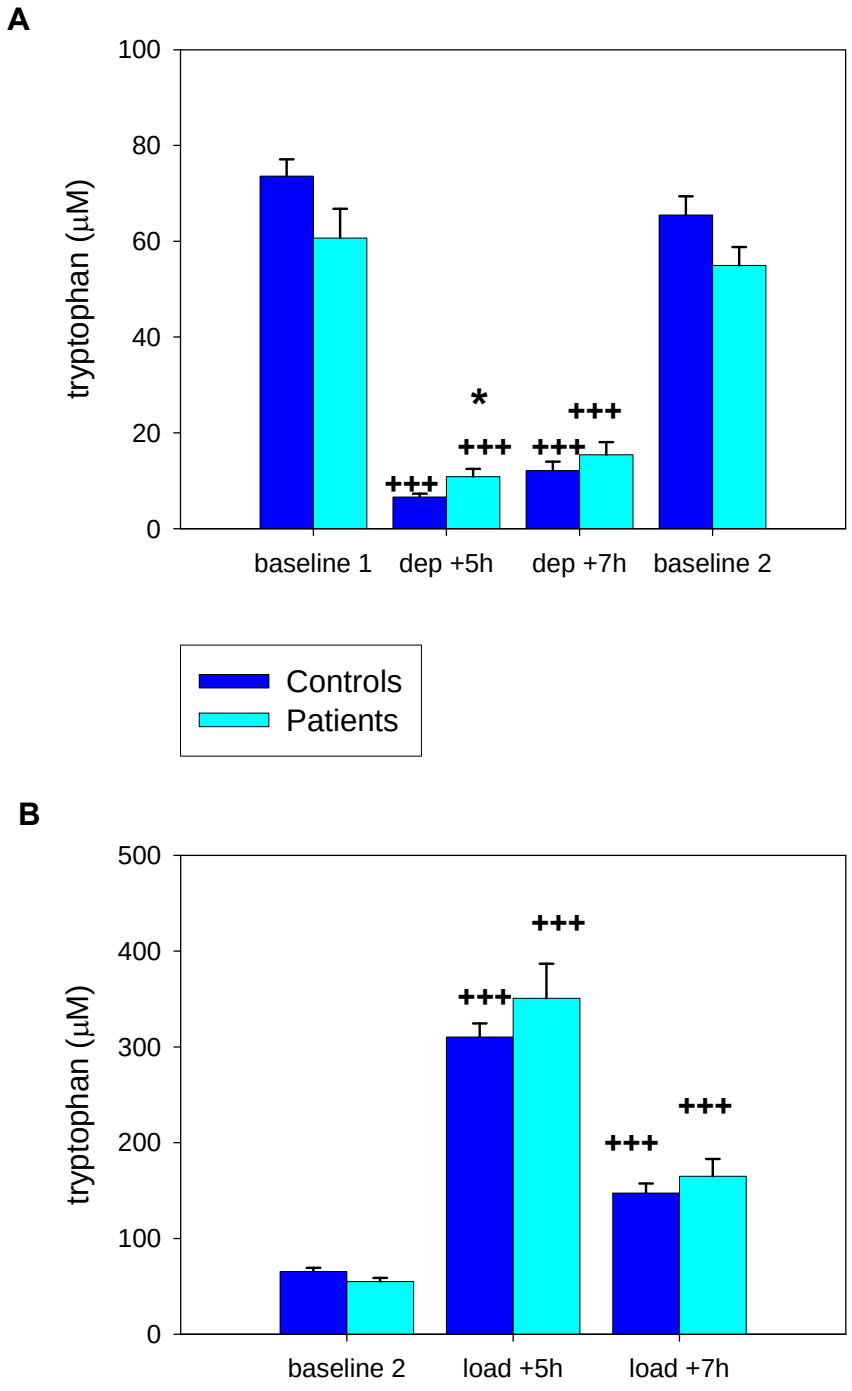


Figure 4-2 Effect of tryptophan depletion and loading on tryptophan levels in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: +++ $p<0.001$.

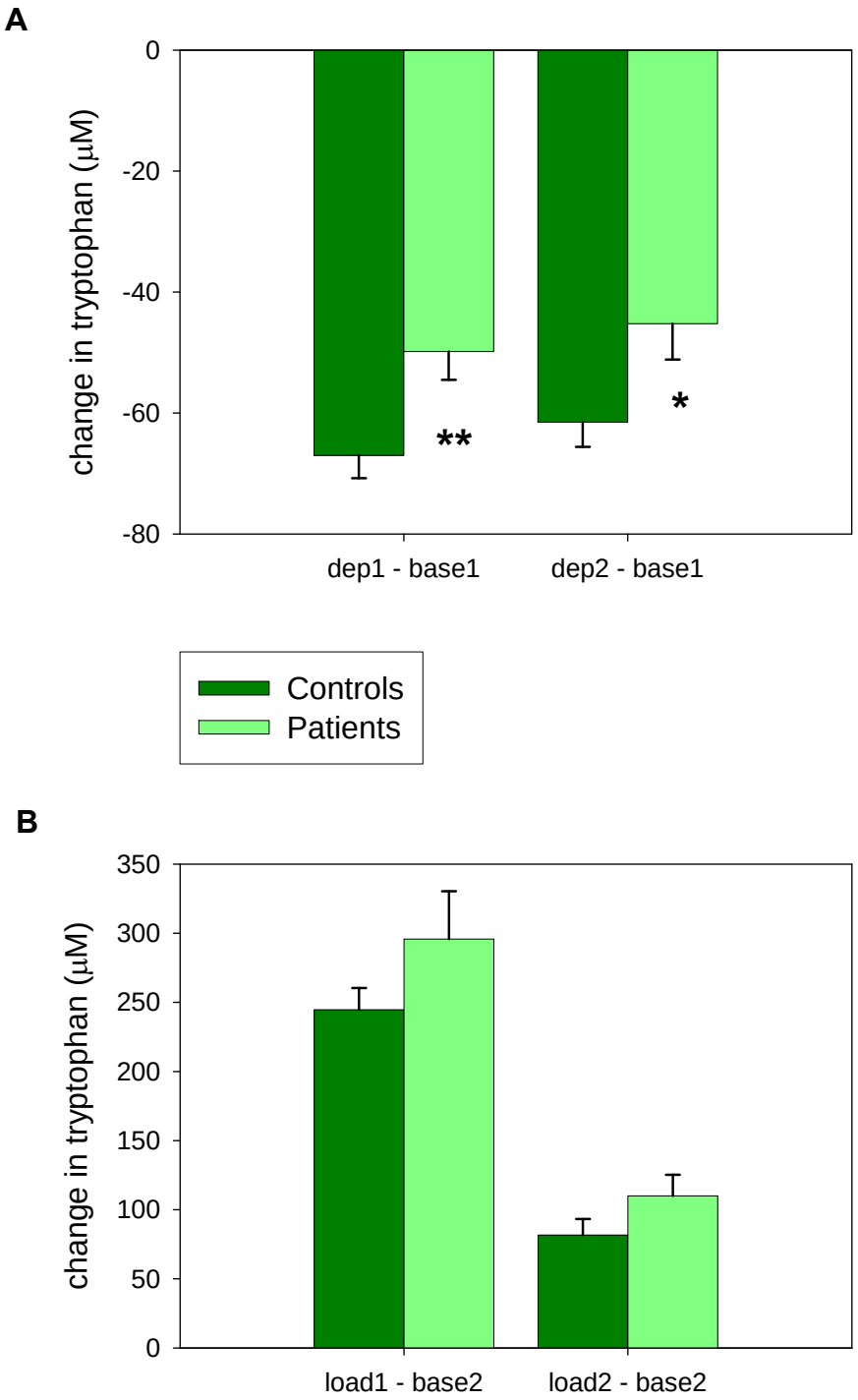


Figure 4-3 Changes in the levels of tryptophan following tryptophan depletion or loading for plasma from HD patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$.

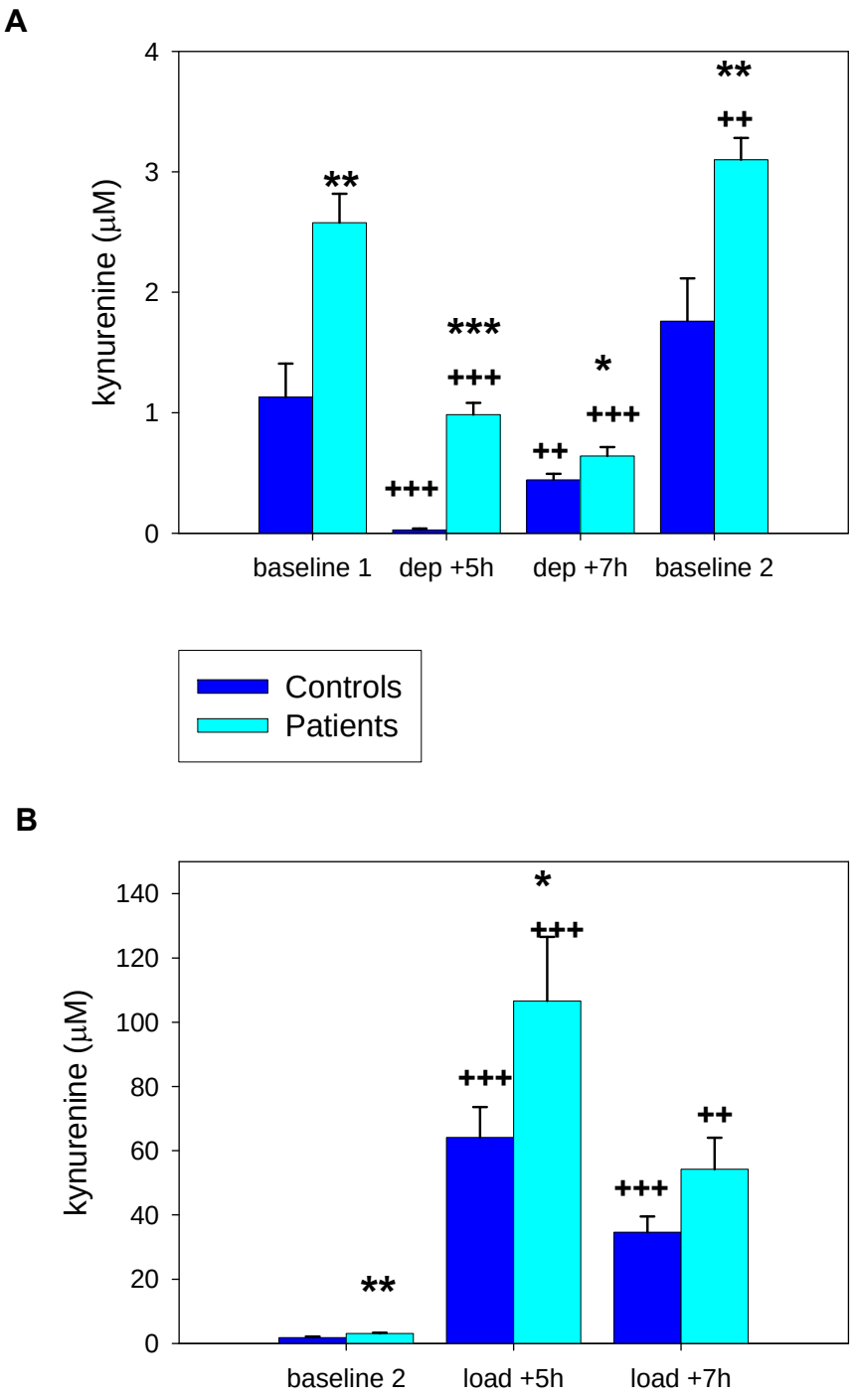


Figure 4-4 Effect of tryptophan depletion and loading on kynurenine levels in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: ++ $p<0.01$, +++ $p<0.001$.

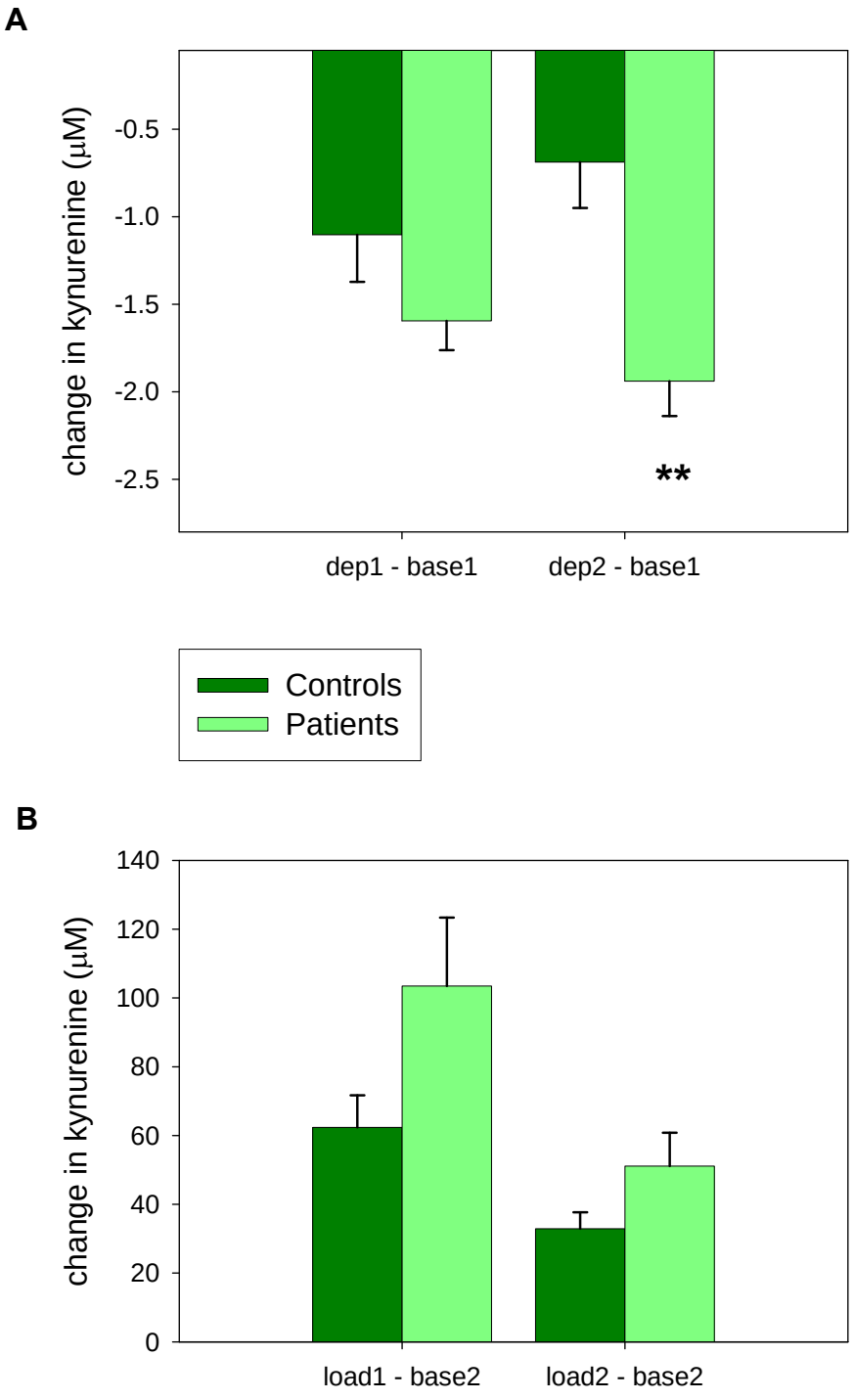


Figure 4-5 Changes in the levels of kynurenine following tryptophan depletion or loading for plasma from HD patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: ** $p<0.01$.

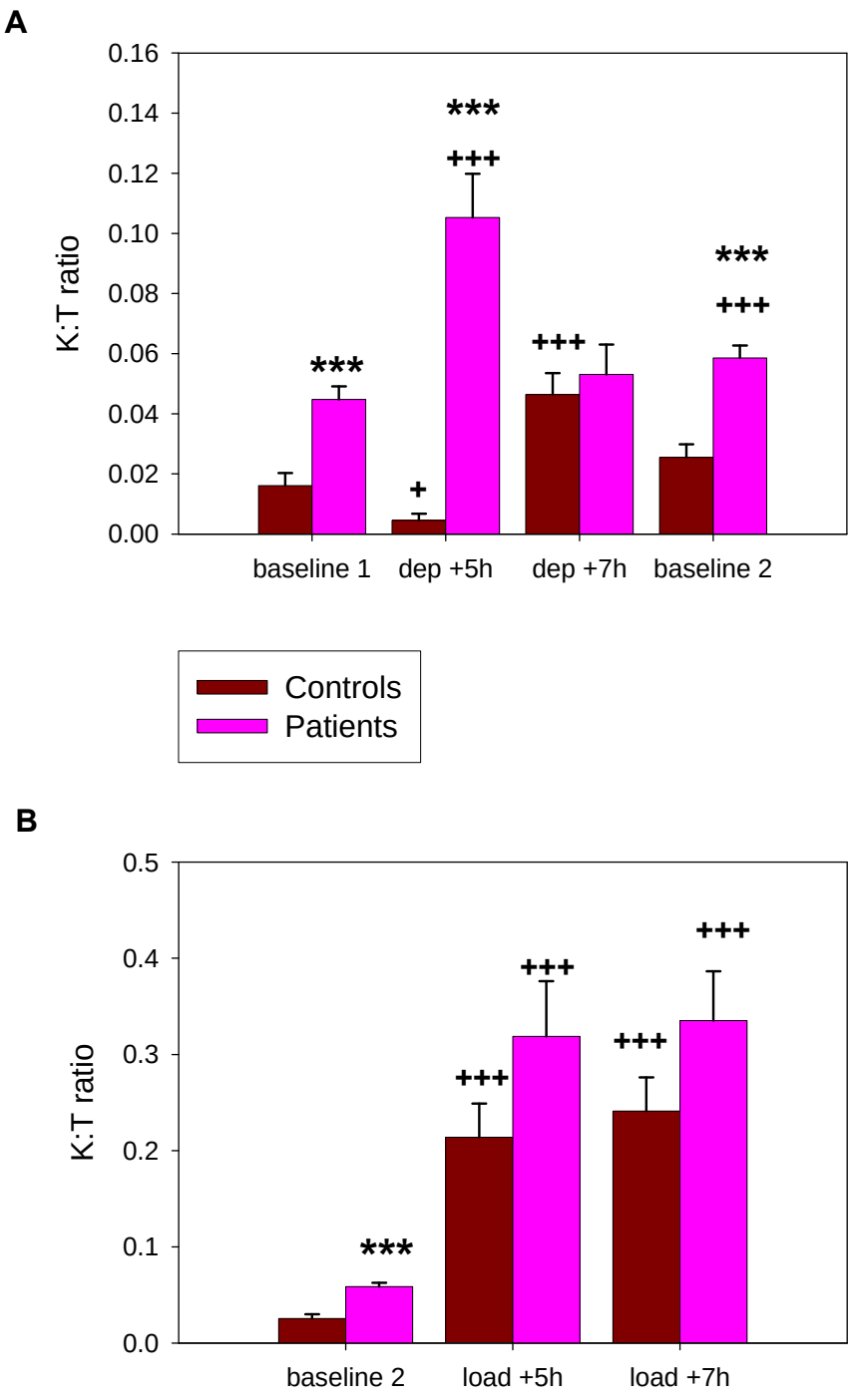


Figure 4-6 Effect of tryptophan depletion and loading on kynurenine:tryptophan (K:T) ratio in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: *** $p< 0.001$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: + $p<0.05$, +++ $p<0.001$.

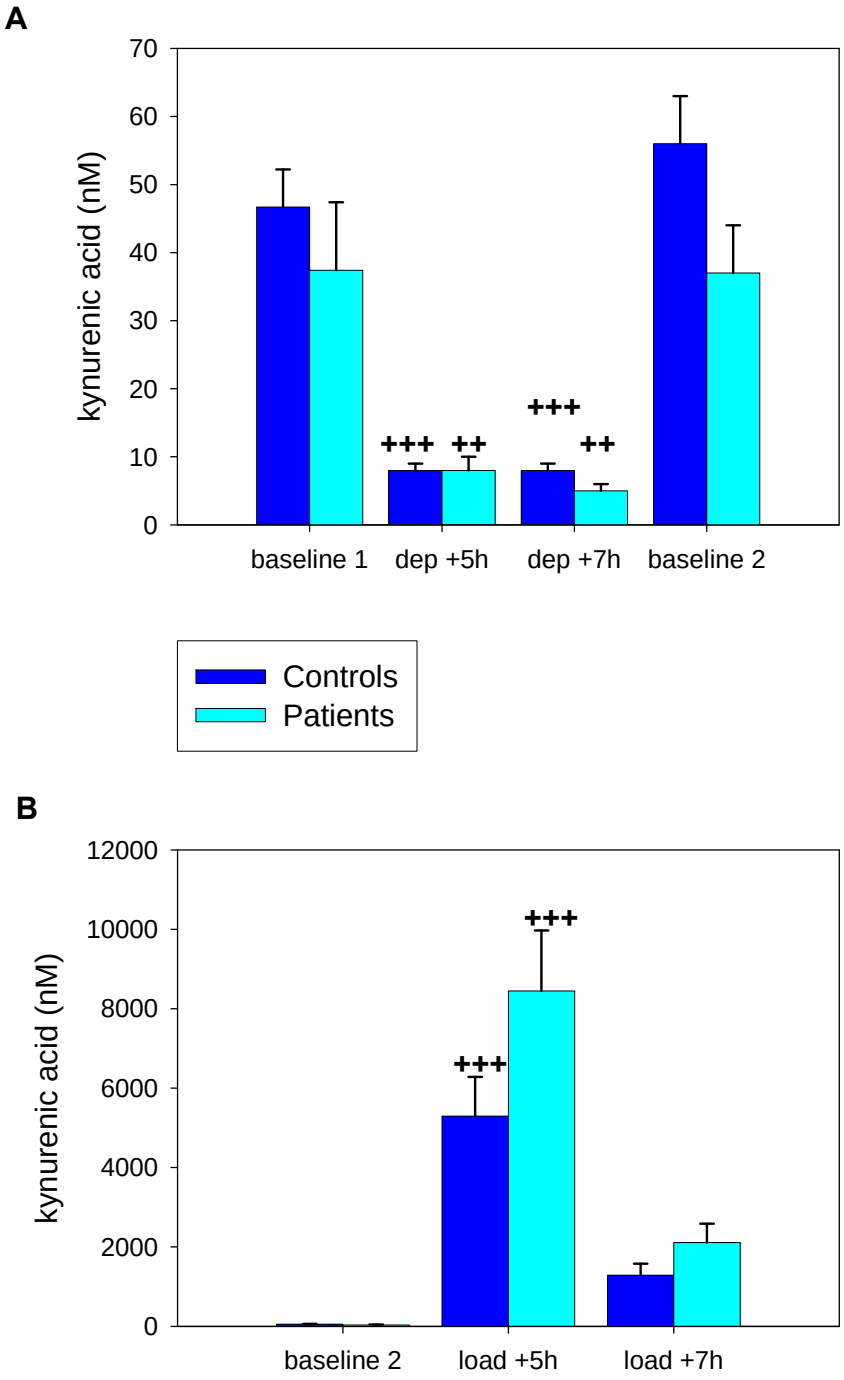


Figure 4-7 Effect of tryptophan depletion and loading on kynurenic acid levels in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. No significant differences between patients and controls. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: **++** $p<0.01$, **+++** $p<0.001$.

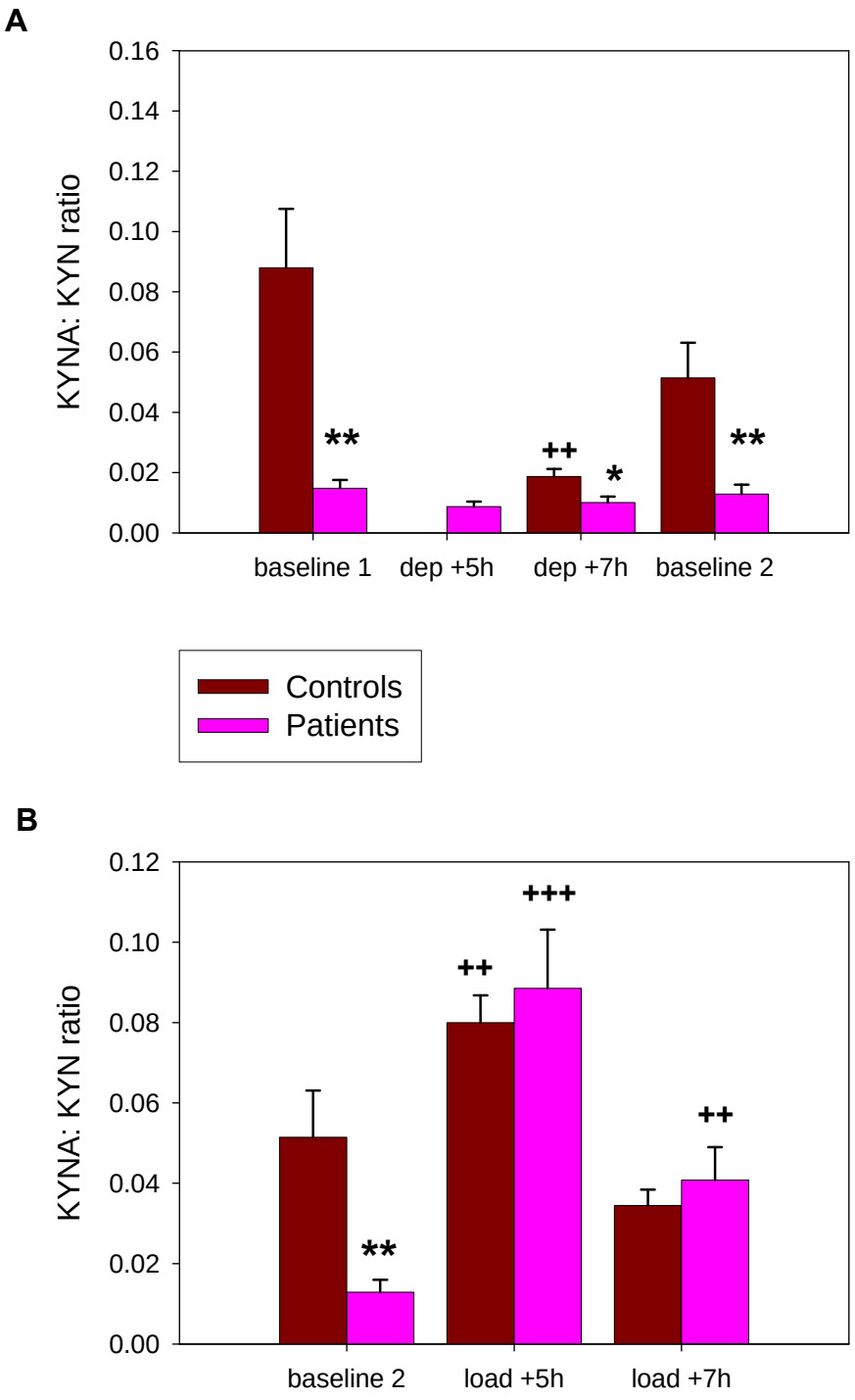


Figure 4-8 Effect of tryptophan depletion and loading on kynurenic acid: kynurenine ratio (KYNA: KYN) in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: ++ $p<0.01$, +++ $p<0.001$. (Many control kynurenine levels were 0 at 5h after depletion and KYNA: KYN ratios could not be calculated, therefore were omitted from the graph.)

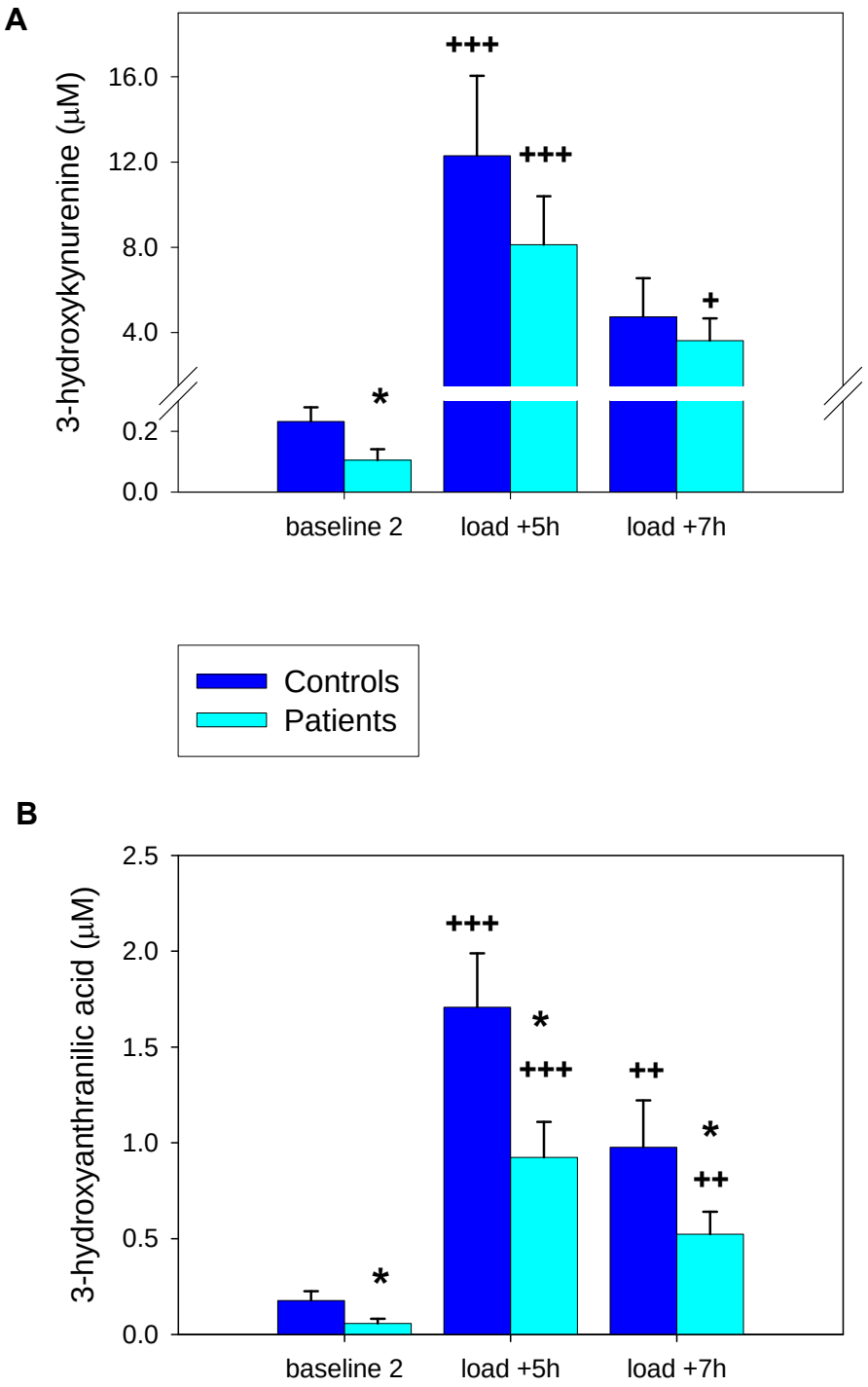


Figure 4-9 Effect of tryptophan loading on the levels of 3-hydroxykynurenine (A) and 3-hydroxyanthranilic acid (B) in the plasma of controls and HD patients at baseline 2 (24h after depletion) and at 5 and 7 hours after loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$. Effect of loading compared with baseline 2: + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

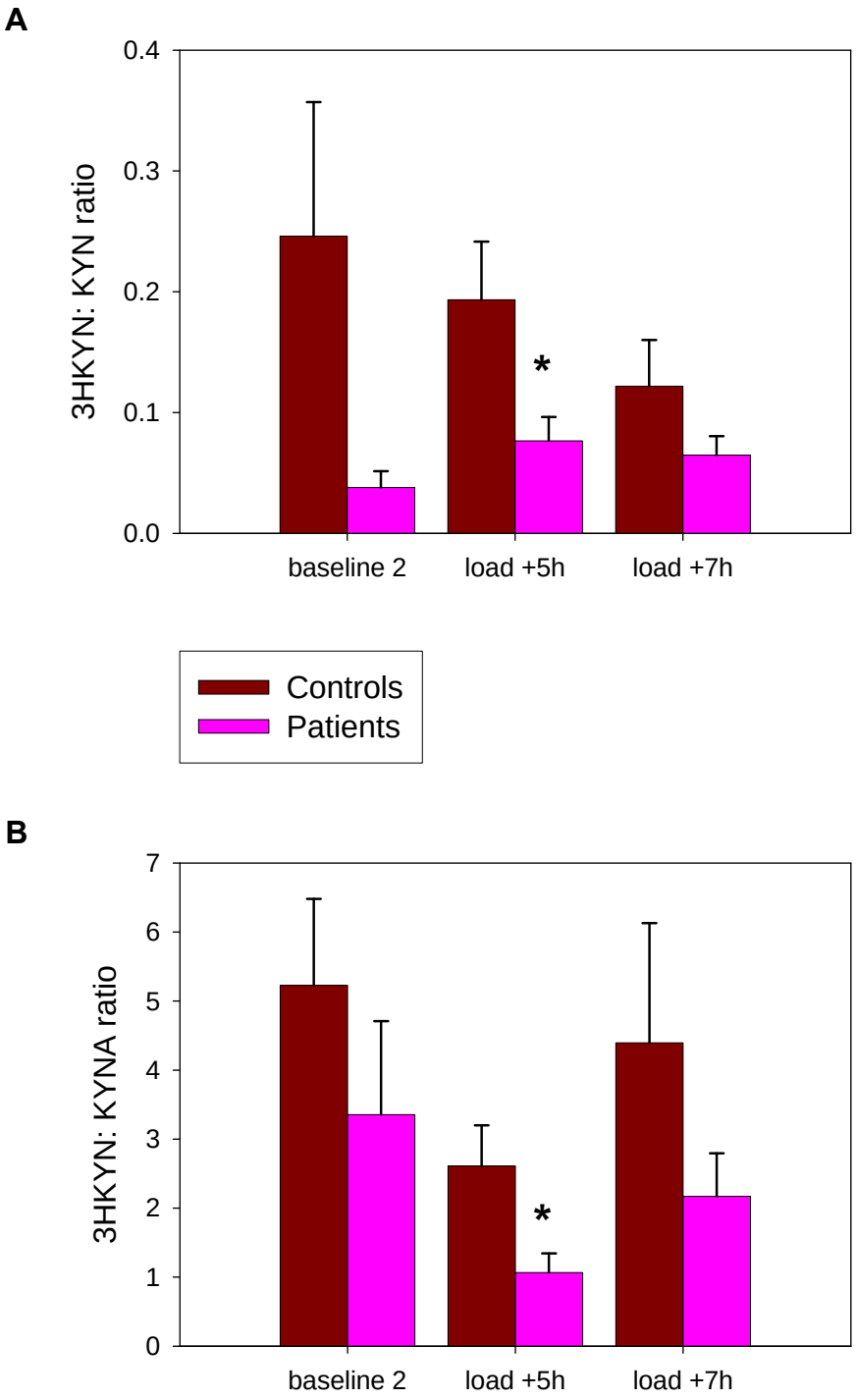


Figure 4-10 Effect of tryptophan loading on 3-hydroxykynurenine: kynurenine (3HKYN: KYN) ratio (A) and 3-hydroxykynurenine: kynurenic acid (3HKYN: KYNA) ratio (B) in the plasma of controls and HD patients. Values are mean ± SEM. Significant differences between patients and controls: * $p < 0.05$.

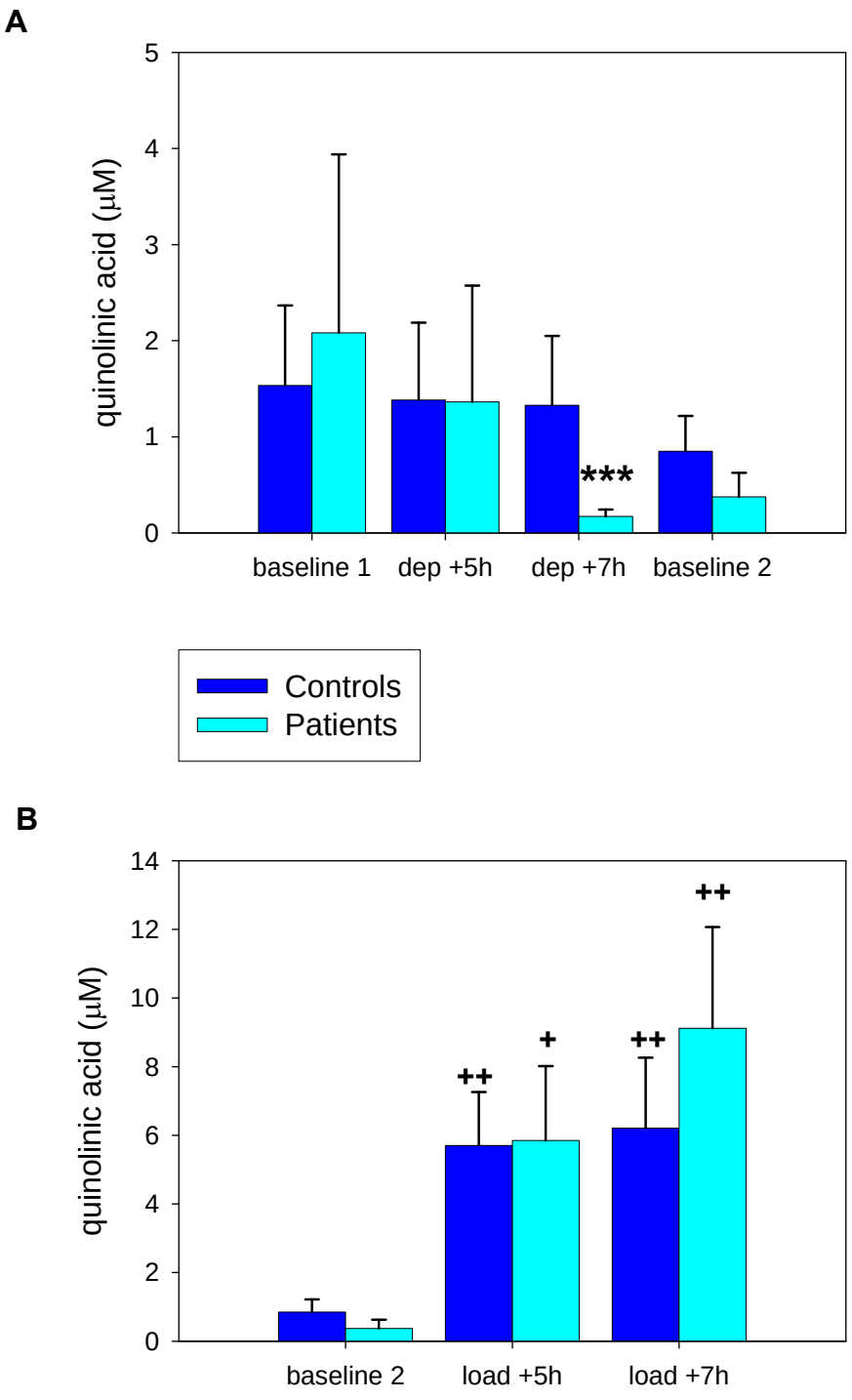


Figure 4-11 Effect of tryptophan depletion and loading on the levels of quinolinic acid in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: *** $p<0.001$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: + $p<0.05$, ++ $p<0.01$.

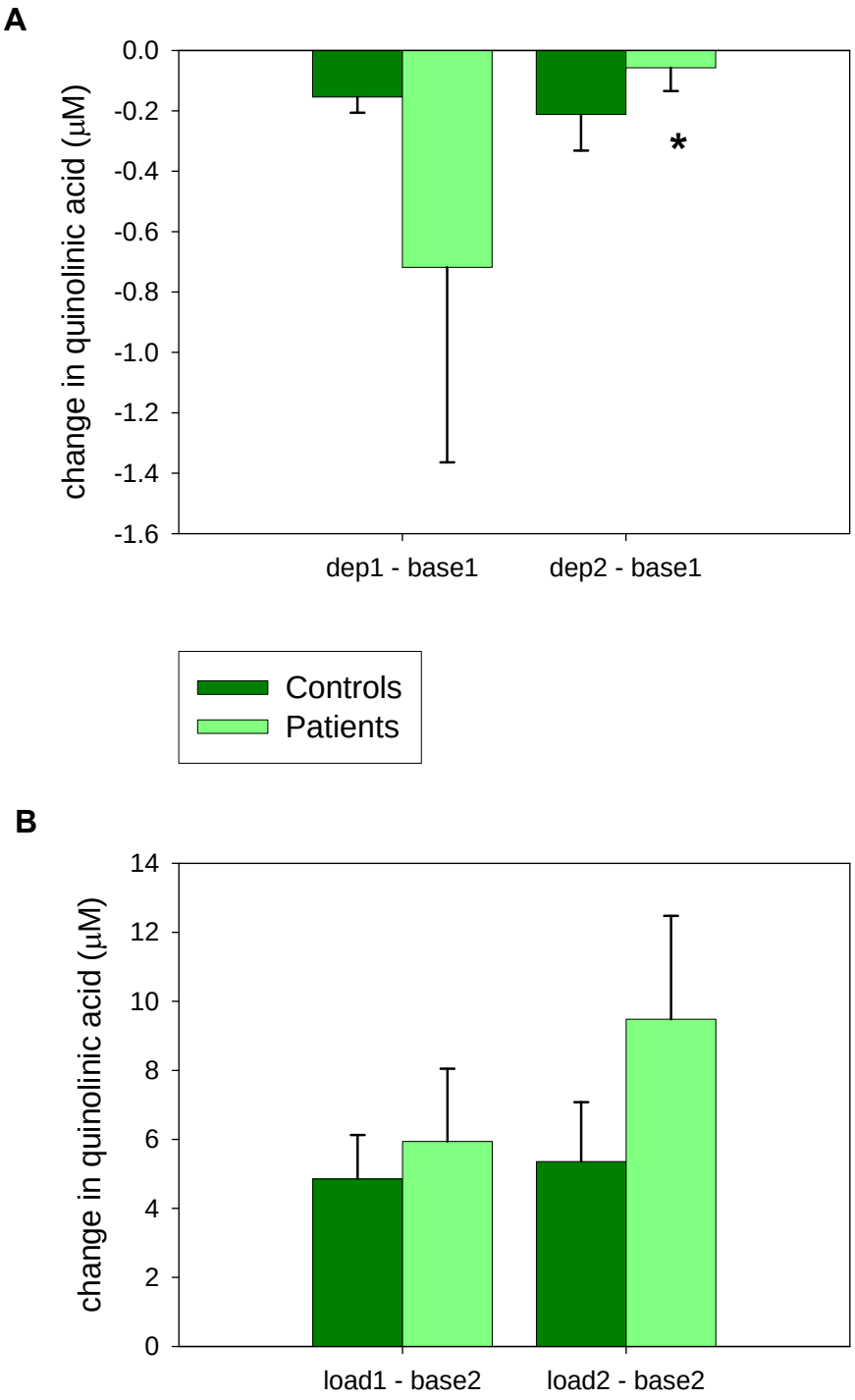


Figure 4-12 Changes in the levels of quinolinic acid following tryptophan depletion or loading for plasma from HD patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: * $p<0.05$.

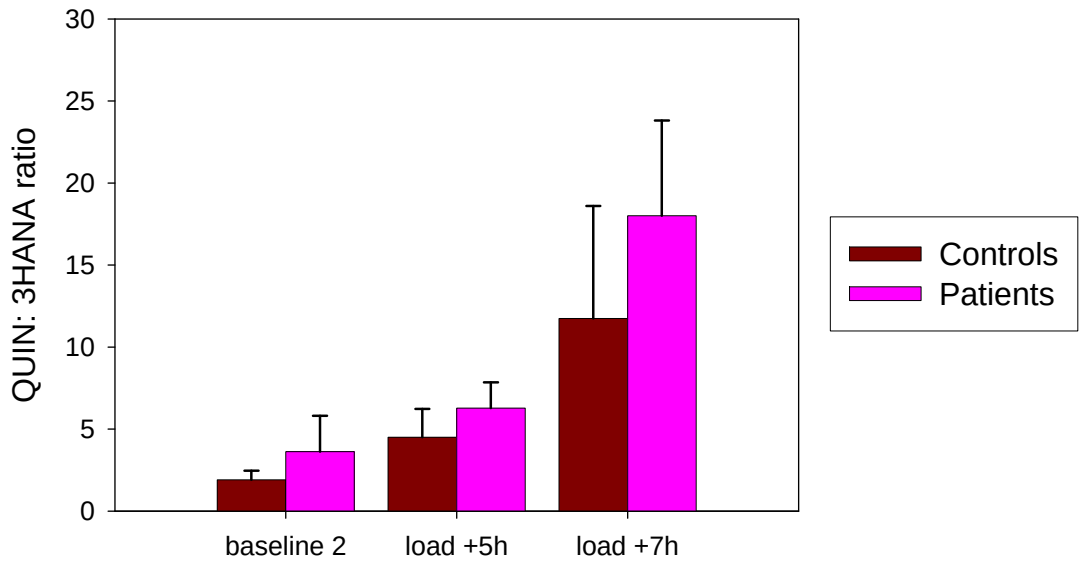


Figure 4-13 Effect of tryptophan loading on quinolinic acid: 3-hydroxyanthranilic acid ratio (QUIN: 3HANA) in the plasma of controls and HD patients. Values are mean ± SEM. No significant differences between patients and controls.

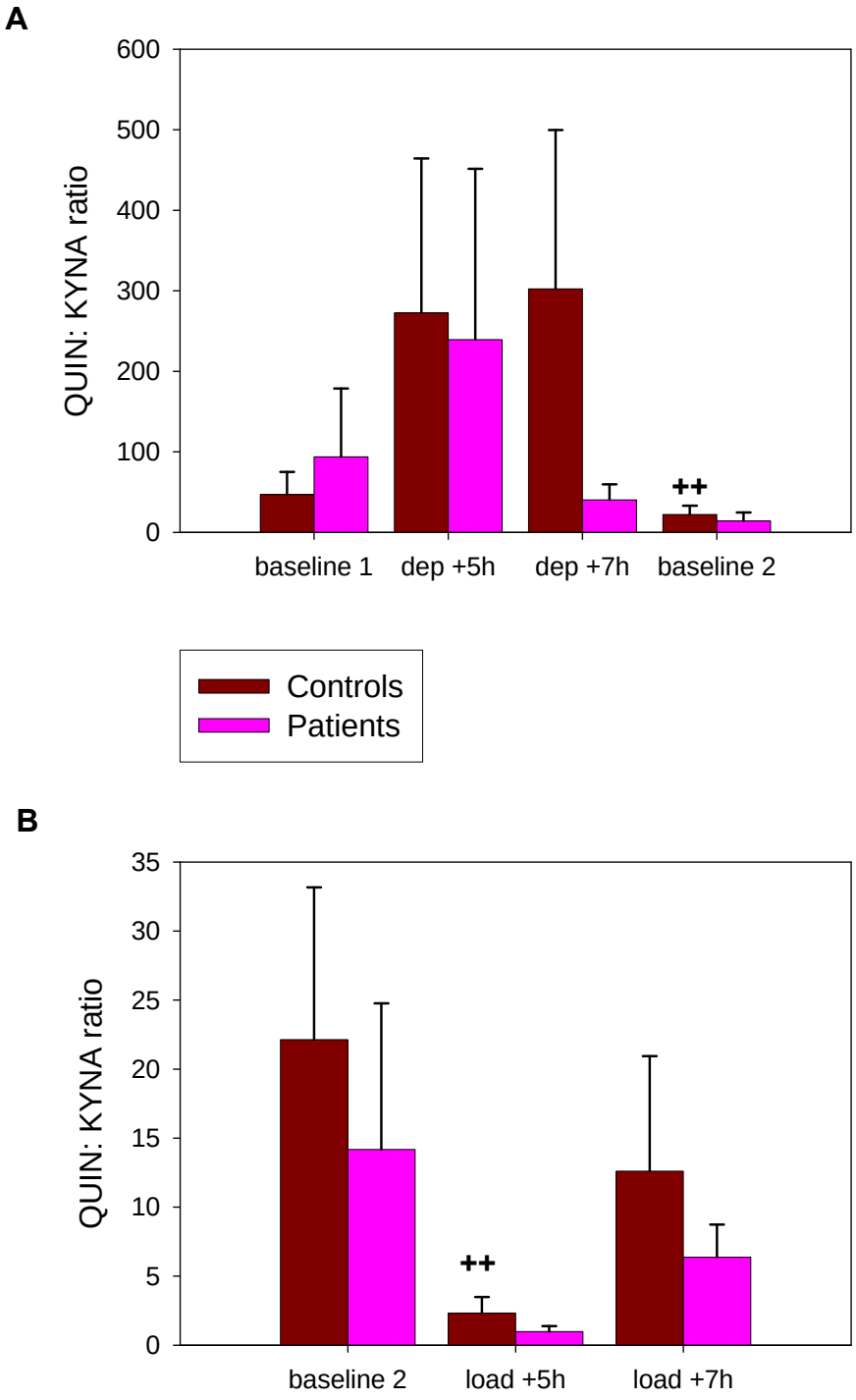


Figure 4-14 Effect of tryptophan depletion and loading on quinolinic acid: kynurenic acid ratio (QUIN: KYNA) in the plasma of controls and HD patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. No significant differences between patients and controls. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: **++** $p<0.01$.

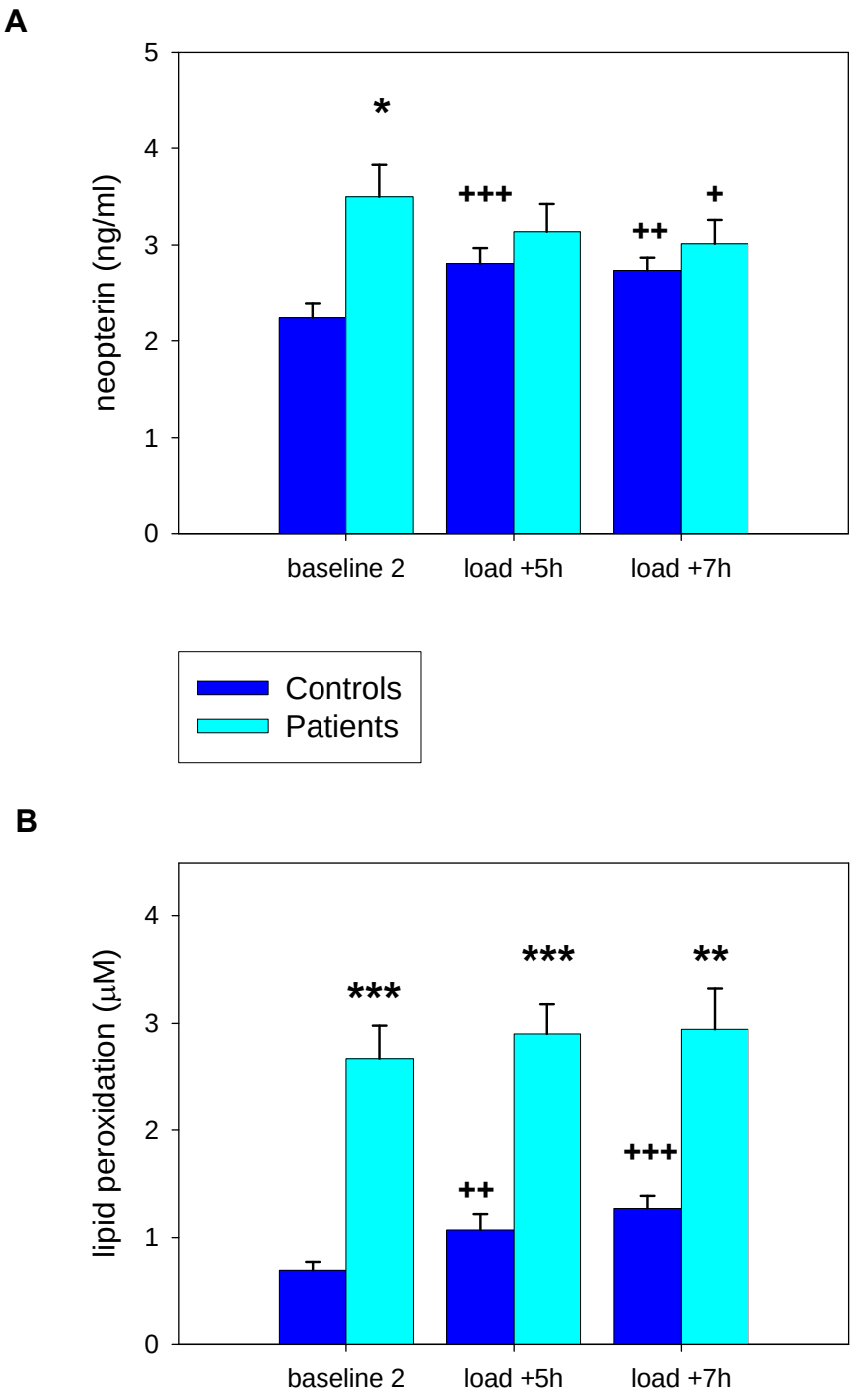


Figure 4-15 Effect of tryptophan loading on the levels of neopterin (A) and lipid peroxidation products (B) in the serum of controls and HD patients at baseline 2 (24h after depletion) and at 5 and 7 hours after loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effect of loading compared with baseline 2: **+** $p<0.05$, **++** $p<0.01$, **+++** $p<0.001$.

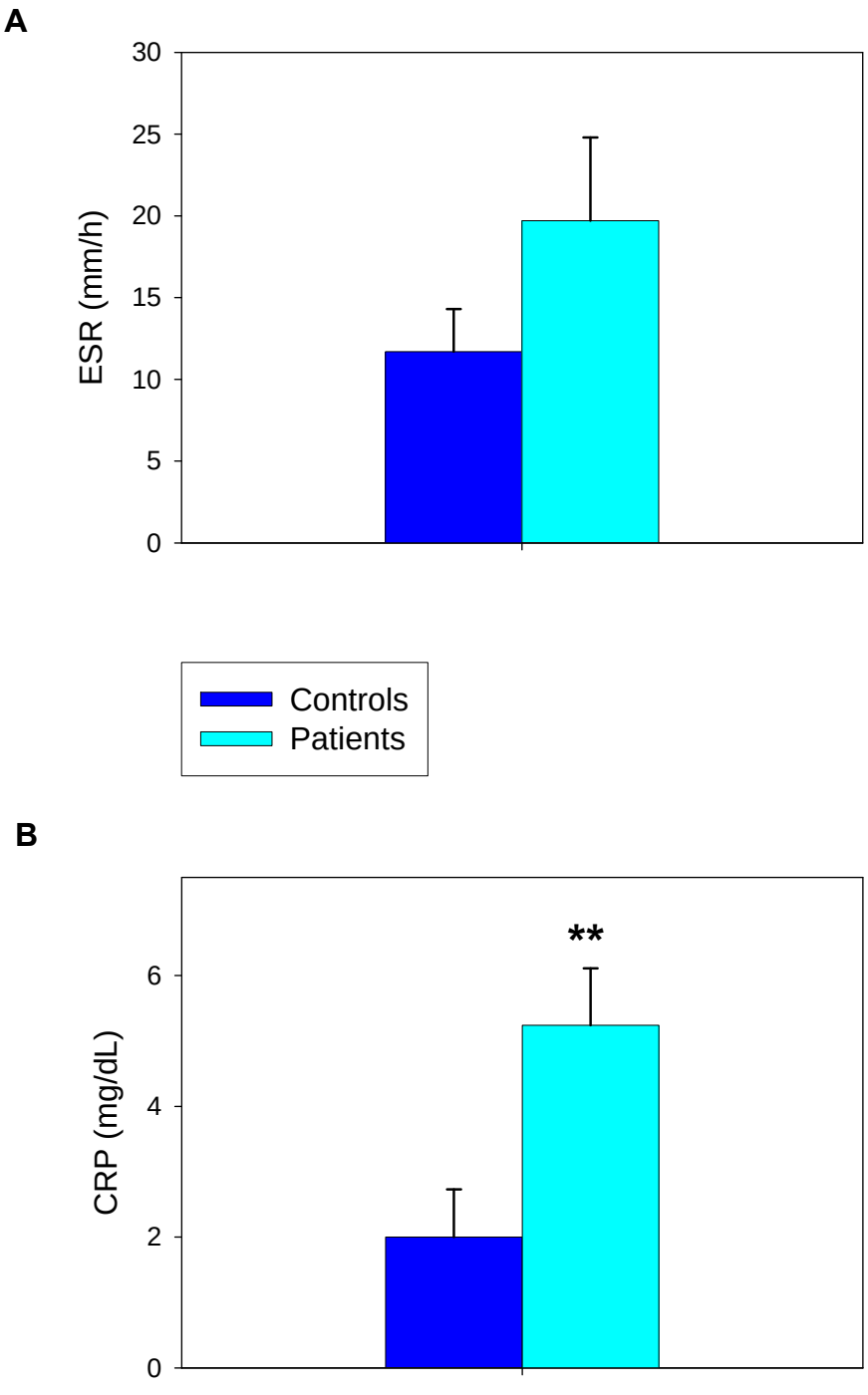


Figure 4-16 Comparison of ESR and CRP between HD patients and controls at baseline. Values are mean ± SEM. Significant differences: ** $p<0.01$.

Table 4-1 Changes in the levels of kynurenines following tryptophan depletion. Values are mean ± SEM. Comparisons between HD patients and controls: not significant: ns.

	Dep+5h - Base1		Dep+7h - Base1	
	Controls	**Patients**	**Controls**	**Patients**
Kynurenic acid (nM)	-35.9 ± 4.8	-29.5 ± 9.4 ns	-36.2 ± 4.8	-32.2 ± 9.6 ns

Note: Changes for tryptophan, kynurenine and quinolinic acid are shown graphically.

Table 4-2 Changes in the levels of kynurenines following tryptophan loading. Values are mean ± SEM. Comparisons between HD patients and controls: not significant: ns.

	Load+5h - Base2		Load+7h - Base2	
	Controls	**Patients**	**Controls**	**Patients**
Kynurenic acid (nM)	5237 ± 988	8409 ± 1525 ns	1227 ± 291	2073 ± 473 ns
3-Hydroxykynurenine (µM)	12.1 ± 3.8	8.0 ± 2.3 ns	4.5 ± 1.8	3.5 ± 1.0 ns
3-Hydroxyanthranilic acid (µM)	1.53 ± 0.28	0.87 ± 0.18 ns	0.80 ± 0.25	0.47 ± 0.12 ns

Note: Changes for tryptophan, kynurenine and quinolinic acid are shown graphically.

4.4 Discussion

4.4.1 Tryptophan Depletion and Loading

The tryptophan depletion and loading protocols produced the desired effects of significantly reducing or increasing, respectively, the tryptophan levels in the bloods of both HD patients and healthy control subjects, at both 5 and 7 hours after the depleting or loading mixtures were administered. Tryptophan levels at 24 hours after depletion had returned to baseline levels for both patients and controls. Tryptophan depletion also reduced the levels of kynurenine and KYNA, although QUIN levels appeared to take at least 7 hours or perhaps longer for the reduction to occur, with levels at 24 hours after depletion (baseline 2) still appearing to be lower than at the initial baseline. However, there were no significant differences in these QUIN levels due to the large variability in the results. For tryptophan, kynurenine, KYNA, 3HKYN and 3HANA, tryptophan loading produced increased levels after 5 hours with levels starting to reduce back to normal after 7 hours. With QUIN, the levels at 7 hours after loading appeared to be similar or perhaps even increasing in patients compared with 5 hours after loading. Unfortunately, no further blood samples were taken at a later time point to investigate whether QUIN levels continued to rise in the patients with HD.

4.4.2 Tryptophan, Kynurenine and IDO/TDO activity

While there was no significant difference in baseline levels of tryptophan between patients with HD and controls in this study, the much higher baseline levels of kynurenine in the patients demonstrated increased metabolism via the kynurenine pathway. These high kynurenine levels resulted in higher K:T ratios in the patients' baseline samples, also indicating enhanced IDO/TDO activity. Increased kynurenine pathway activity was observed throughout the depletion and loading protocols as the kynurenine levels remained significantly higher in the patients at 5 of the 6 time points. IDO activity may be upregulated in the HD patients due to the increased inflammation observed, as the levels of the inflammatory marker, neopterin, was significantly higher in patients than in controls at baseline, as was the level of CRP. Indeed, there was a significant correlation between neopterin and the K:T ratio. In human macrophages, stimulation by the pro-inflammatory cytokine, IFN-γ, activates IDO and in parallel, induces the enzyme, GTP-cyclohydrolase I, which enhances neopterin formation (Schroecksnadel *et al.*, 2005). The inflammatory response causes the activation of macrophages and microglia, both of which express IDO

(Heyes *et al.*, 1996). In addition, the increased oxidative stress, shown by the increase in lipid peroxidation products in this study, may result in the production of superoxide in HD patients, which would contribute to stimulating IDO activity as superoxide acts as a co-substrate for IDO.

At 5 hours after depletion, patients showed less reduction in tryptophan levels than controls, although there was more IDO/TDO activity (higher kynurenine and K:T ratios). This suggests that patients require less tryptophan for other metabolic processes, and hence depleting tryptophan has less of an effect on blood tryptophan levels. Depletion did not reduce the IDO/TDO activity in patients, reflected by the K:T ratios, which remained high at 5 and 7 hours after depletion. With more available tryptophan after loading, IDO/TDO activity increased in patients and also in controls. As IDO is not expected to be very active in controls, much of this increase in K:T ratios on loading may be due to TDO activity in the liver. Indeed, tryptophan is known to stimulate TDO activity (Schutz *et al.*, 1972). The level of kynurenine was still significantly higher in patients than in controls at 5 hours after loading, suggesting that even after loading, IDO activity still appears to be contributing to the activation of the kynurenine pathway in patients.

4.4.3 Kynurenic Acid and KAT activity

There are conflicting reports on the levels of KYNA in the brain of HD patients. Connick *et al*. (1989) showed increased KYNA in the motor cortex of post-mortem HD brains, with no differences in KYNA levels in the prefrontal cortex, the caudate nucleus or globus pallidus compared to controls. Reduced concentrations of KYNA were demonstrated in the CSF of HD patients (Beal *et al.,* 1990) and in HD cerebral cortex (Beal *et al.,* 1992). In addition, a significant increase in the ratio kynurenine: KYNA was demonstrated in HD post-mortem brains (Beal *et al.,* 1990). Out of 12 brain regions from late-stage HD brains, the only region of the brain that showed a significant reduction in the level of KYNA was the caudate nucleus (Jauch *et al*., 1995). The putamen and globus pallidus also suggested reduced KYNA levels but these were not significant. These three regions are all part of the striatum, which is the brain region most susceptible to neuronal damage in HD, with the cortex being the next most susceptible region. It was hypothesised that if KYNA regulates by blocking NMDA receptor activation, a chronic deficiency could potentially lead to a slowly developing NMDA excitotoxic neurodegenerative process (Beal *et al*., 1990). However, the reported concentrations of KYNA required to block NMDA receptor activation are about 100-fold greater than those found in the human brain (Jahr and Jessel, 1985).

In this study, the levels of KYNA were similar for patients and controls throughout the depletion and loading protocols. However, when the ratios of KYNA: kynurenine (KYNA: KYN) were compared, these were lower for the patients at baseline and following depletion, demonstrating that the much higher levels of kynurenine in patients could not produce increased levels of KYNA. In addition, negative correlations were observed between the K:T ratio and KYNA at both time points after depletion. In contrast, the healthy controls showed a positive correlation between K:T ratio and KYNA at the first baseline. This suggests that the KAT enzymes are not functioning optimally in the patients. Neopterin also correlated negatively with KYNA: KYN ratio at baseline in HD patients, suggesting that the inflammatory response may inhibit KAT activity. There is no clear evidence for a decrease in KAT activity in HD brains. Jauch *et al*. (1995) showed that the only brain region out of the 12 regions examined from HD brains, with significantly reduced KAT I and KAT II activities was the putamen, which also had approximately 3-fold higher Km for both enzymes. Similarly, Pearson *et al*. (1995) found no change in KAT activity in HD brain in the caudate or frontal cortex. However, on tryptophan loading in our study, the KYNA: KYN ratios are similar for patients and controls, with the patients actually having a mean level slightly higher than the controls, showing that the activity of the KAT enzymes has increased in the patients to levels similar to the controls. This suggests that tryptophan loading may offer neuroprotection to HD patients due to their ability to produce increased amounts of KYNA.

4.4.4 3-Hydroxykynurenine, 3-Hydroxyanthranilic Acid and Oxidative Stress

3HKYN and 3HANA have the potential to cause neuronal damage by generating reactive oxygen species such as hydrogen peroxide, with the resulting oxidative stress inducing apoptosis (Eastman and Guilarte, 1989; Eastman and Guilarte, 1990; Okuda *et al*., 1996; Okuda *et al*., 1998). The highly significant increases in lipid peroxidation observed in baseline and loaded patients' samples demonstrate increased oxidative stress in the HD patients. Levels of 3HKYN and 3HANA were significantly lower in the bloods of patients compared with controls at baseline. For 3HANA, patients also had lower levels at both 5 and 7 hours after loading. In addition, controls, but not patients, showed positive correlations between K:T ratio and 3HANA at both 5 and 7 hours after loading, suggesting that normal kynurenine pathway metabolism would increase the levels of 3HANA in the blood. Furthermore, when the ratios of 3HKYN: kynurenine and 3HKYN: KYNA were examined, they were both significantly lower at 5 hours after loading. This may suggest

that 3HKYN and 3HANA are being utilised, perhaps being autoxidised resulting in the formation of reactive oxygen species. The resulting products of several oxidation steps in autoxidation produce hydrogen peroxide and superoxide (Dykens *et al*., 1987; Ishii *et al*., 1992; Hiraku *et al*., 1995). The superoxide could induce further kynurenine pathway metabolism as IDO requires superoxide as a co-substrate. These results are the opposite to the findings of Pearson and Reynolds (1992) and Guidetti *et al.* (2000), where 3HKYN levels were shown to be increased in post-mortem HD brain and the ratio of 3HKYN: KYNA increased. Although in some studies 3HKYN levels in HD brains were not significantly increased (Beal *et al.*, 1990), there was no evidence for decreased levels of brain 3HKYN in HD patients. This study measured peripheral levels rather than those in the brain. As both kynurenine and 3HKYN can cross the blood-brain barrier using the large neutral amino acid transporter (Fukui *et al*., 1991), the levels of 3HKYN in the brain may still be higher in the HD patients. The areas of the brain most affected by HD, the striatum and cerebral cortex, are where elevated levels of 3HKYN have been shown to be present and also have the highest capacity for 3HKYN uptake. As 3HKYN is metabolised in the brain either by autoxidising or producing 3HANA, 3HKYN uptake from the blood may increase, thus depleting the 3HKYN and the production of 3HANA in the blood.

Alternatively, as 3HKYN and 3HANA can autoxidise, the initial oxidation can, depending on the presence of other redox-active species, allow them to act as antioxidants (Christen *et al*., 1990; Leipnitz *et al*., 2007). The lower levels of 3HKYN and 3HANA in the blood of HD patients in this study may reduce their ability to act as free radical scavengers and hence these compounds are unable to control the increased oxidative stress in these patients. This may be due to less 3HKYN being produced, which in turn would produce less 3HANA. The lower 3HKYN production could contribute to the increased kynurenine levels observed and may suggest inhibition of the enzyme, KMO, which produces 3HKYN from kynurenine.

4.4.5 Quinolinic Acid

A hypothesis that has been developed over the past 20 years is that the mechanism for neuronal loss in HD is an excessive stimulation of NMDA receptors by QUIN. This hypothesis has been supported by QUIN injections into the brain producing very similar lesions to those observed in HD, in particular the selective sparing of somatostatin/ neuropeptide Y neurones in the striatum (Beal *et al*., 1986; Beal *et al.,* 1989) and also as cells with high densities of NMDA receptors are preferentially lost in HD striatum (Young *et al*., 1988). However, only recently has an increase in QUIN been observed in HD post-

mortem brains and this was for patients with early grade HD (Guidetti *et al*., 2004). In the same study, QUIN levels were not elevated in brain tissue from at-risk patients, or patients with grades 2, 3 or 4 HD.

In this study, there was much variability in the levels of QUIN and the only significant difference between patients and controls was a decrease in QUIN levels for patients at 7 hours after depletion. To try to reduce the effect of the high variability in QUIN levels within both the patient and control groups, the changes in QUIN levels on depletion or on loading were calculated and compared, but this did not result in any additional significant effects. Little difference in blood QUIN concentrations between patients with late stage HD and controls is in agreement with previous studies with brain tissue from later grades of HD which showed that there was no increase in QUIN levels in these brains or CSF (Reynolds *et al*., 1988; Schwarcz *et al*., 1988b; Heyes *et al*., 1991b). Heyes *et al*. (1991b) actually showed reduced levels of QUIN, significantly lower in some areas of the cerebral cortex. It is unlikely that prevention of QUIN production in HD patients is caused by insufficient activity of the enzyme, 3HAO, which converts 3HANA into QUIN, as its activity has been shown to be increased in HD brain (Schwarcz *et al*., 1988a). The present results actually suggest that there may be increased 3HAO activity, as the lower baseline levels of 3HANA in patients produce similar levels of QUIN compared to controls. When the ratios of QUIN: 3HANA were calculated, the differences between patients and controls were not significant but the trend at both baseline and after loading suggested higher amounts of QUIN may be produced from 3HANA in the patients.

4.4.6 Inflammation: Neopterin, ESR and CRP

Levels of neopterin and CRP were significantly increased at baseline in HD patients compared to controls, consistent with evidence of activated microglia in HD (Sapp *et al*., 2001). Inflammation and raised levels of neopterin are often associated with increased activity of reactive oxygen species (Murr *et al*., 2002) and although both neopterin levels and lipid peroxidation were increased in the HD patients, no correlations between them were observed. The raised serum neopterin levels in HD patients is consistent with the study of Leblhuber *et al*. (1998), where increased neopterin and decreased tryptophan in HD patients were associated with loss of cognitive function. The correlation between neopterin and the K:T ratio at baseline is in agreement with observations in other human diseases and suggests that the pro-inflammatory cytokine, IFN-γ, has activated both neopterin and IDO (Schroecksnadel *et al*., 2005).

4.4.7 Lipid Peroxidation and Oxidative Stress

It has been proposed that mitochondrial dysfunction plays a role in the pathogenesis of HD. Oxidative damage is one of the major consequences of defects in energy metabolism. There is evidence of oxidative damage in HD brain (Browne *et al*., 1999; Polidori *et al*., 1999) and in animal models of HD (Perez-Severiano *et al*., 2000; Bogdanov *et al*., 2001).

The lipid peroxidation was significantly higher in HD patients than in controls, showing that oxidative stress was present in HD patients and remained elevated with tryptophan loading. There were no correlations between the kynurenines and lipid peroxidation levels. In particular, there were no correlations with the potential generators of reactive oxygen species, 3HKYN, 3HANA and QUIN. This may suggest that 3HKYN and 3HANA are not contributing to lipid peroxidation, although it must be recognised that the correlations may not be significant due to the small number of patients in the study.

4.4.8 Tryptophan Loading and Oxidative Stress

An unexpected result was that tryptophan loading increased the lipid peroxidation levels in the control subjects. Levels had increased significantly from baseline at both time points after loading. Tryptophan loading induces TDO activity (Schutz *et al*., 1972), and TDO activity in the liver can account for the high levels of the kynurenines produced in the blood of control subjects. The increased levels of QUIN after loading could have activated NMDA receptors, which induces free radical formation. This may have been limited as the NMDA receptor antagonist, KYNA, was also significantly increased after tryptophan loading. In addition, the increased oxidative stress may be due to the high levels of 3HKYN, 3HANA and QUIN directly generating reactive oxygen species, including superoxide, hydrogen peroxide and the hydroxyl free radical (Goda *et al*., 1996; Goldstein *et al*., 2000).

However, there was no significant increased lipid peroxidation in the HD patients on loading, although levels did increase slightly. As the pathway was already stimulated in the HD patients, mechanisms producing increased oxidative stress were already active and therefore there could be less of a contribution to oxidative stress from the tryptophan load.

In conjunction with the increase in lipid peroxidation, neopterin levels were also significantly increased after tryptophan loading in the healthy controls. An increase in reactive oxygen species contributes to the production of inflammatory mediators and

excitotoxicity which can trigger inflammation (Dirnagl *et al*., 1999), and therefore whether the increased lipid peroxidation was produced by 3HKYN, 3HANA and/or QUIN directly generating free radicals or by QUIN's excessive activation of the NMDA receptors, it is not surprising that the neopterin levels were also increased after tryptophan loading. This increase in inflammation suggests that IDO activity may well contribute to the high levels of kynurenine metabolites produced on loading.

4.4.9 Conclusions

Abnormal metabolism of tryptophan via the kynurenine pathway has been demonstrated in the blood of patients with late stage HD, together with increased inflammation and oxidative stress. In HD, increased activity of the initial enzyme of the pathway, IDO, was associated with inactivity of KAT, the enzyme which produces the neuroprotectant, KYNA. The lower levels of 3HKYN and 3HANA either suggest that less is produced, due to lowered activity of KMO, or that 3HKYN and 3HANA are autoxidising at a higher rate than they can be produced. Alternatively, there may be significant uptake of 3HKYN from the blood into the brain, thus depleting the levels in the blood. The lower levels of 3HKYN and 3HANA did not change the levels of QUIN in HD patients.

The differences in the levels of tryptophan metabolites between patients and controls were similar at baseline and after tryptophan depletion. Depletion of tryptophan therefore did not appear to have a beneficial effect compared to a normal diet. On loading, however, many of these differences were no longer present. Indeed, for the HD patients, there was a change from the apparent inhibition of KAT at baseline (reduced KYNA: KYN ratio) and on depletion to significant KAT activity on loading. This suggests that tryptophan loading may be beneficial for HD patients. However, the results suggest a possible build up of QUIN over time after loading in HD patients and therefore tryptophan loading cannot be justified as a neuroprotective measure.

Tryptophan loading in healthy control subjects resulted in increased inflammation and oxidative stress, suggesting that a tryptophan load may be harmful.

5 Chronic Brain Injury

5.1 Introduction

TBI, particularly when severe, can cause major disruption of brain tissue, and involves excitotoxicity, oxidative stress and inflammation (Sahuquillo *et al*., 2001). Indeed Lenzlinger *et al*. (2001) showed an increase in the inflammatory marker, neopterin, in the CSF and serum of patients during the first week after TBI. Increased levels were maintained in the CSF during the following 2 weeks, but began to decrease in the serum during the third week. Inflammation produces free radicals and cytokines which can activate the kynurenine pathway. Activation of the kynurenine pathway has been suggested as the levels of QUIN in the CSF rose following TBI in humans (Sinz *et al*., 1998). In addition, KYNA greatly reduced hippocampal neuronal loss in a rat model of TBI (Hicks *et al*., 1994).

This study investigated whether the kynurenine pathway continues to be activated in patients with severe chronic brain injury, where the injury was sustained at least one year previously. To study the pathway in more detail, the effects of tryptophan depletion and loading on kynurenine concentrations in blood from clinically stable patients and normal healthy controls were examined. In addition to measuring the kynurenines, the levels of the inflammation marker neopterin and lipid peroxidation products, malondialdehyde and 4-hydroxynonenal, as markers of oxidative stress were also determined. ESR and CRP measurements were also examined.

5.2 Methods

5.2.1 Patients and Sampling

Fifteen patients (9 males, 6 females; age 48 ± 3.8 (mean ± SEM)) with severe chronic brain injury, sustained between 1 and 35 years prior to this study, now in a clinically stable condition, were recruited from the Royal Hospital for Neuro-disability. Patients had sustained brain insults severe enough to require long term care in a specialist brain injury unit, and include 8 involved in road traffic accidents, 2 with head injury, 3 patients who had suffered hypoxia, one with cerebral infarcts and another had a history of a subdural haematoma. There were no clear parameters which distinguished any patient or sub-group of patients from the others, irrespective of the cause of the injury or the duration since the

injury. Fifteen healthy subjects (4 males, 11 females; age 43 ± 2.2 (mean ± SEM)) acting as controls were also enrolled in the study. Patients were recruited after obtaining ethical permission from the Ethical Committee of the Epsom and St Helier NHS Trust and from the Riverside Ethical Committee of the Royal Hospital for Neuro-disability. Informed written consent was obtained from all subjects or their next of kin. None of the patients was on any medication that was known to have any effect on tryptophan or the kynurenines.

The tryptophan depletion and loading protocol was the same for these patients and controls as for the Huntington's patients (Section 4.2.1).

Blood samples were protected from light throughout. EDTA plasma and serum samples were prepared by centrifugation at 1000g for 10 minutes, and frozen within two hours of venesection. Samples were kept frozen while being transported from Epsom General Hospital to the University of Glasgow and then stored at -40ºC prior to analysis.

5.2.2 Kynurenines by HPLC

Samples of EDTA plasma were extracted for HPLC (Section 3.1.2). Mrs R McMillan helped with some of the extractions. Tryptophan, kynurenine and kynurenic acid were analysed by RP-HPLC described in Section 3.2.3, using absorbance and fluorescence detection for baseline, tryptophan depleted and tryptophan loaded samples. 3-hydroxykynurenine, 3-hydroxyanthranilic acid and xanthurenic acid levels were measured in baseline samples and following tryptophan loading using RP-HPLC with electrochemical detection as described in Section 3.5.3.

5.2.3 QUIN by GC-MS

Samples of EDTA plasma were analysed for QUIN by West Park Biochemistry Laboratories by the method described in the appendix for baseline, tryptophan depleted and tryptophan loaded samples.

5.2.4 Neopterin and Lipid Peroxidation

Neopterin and lipid peroxidation were determined by Dr C.M. Forrest at the University of Glasgow using the methods described in the appendix. Only baseline and loaded serum samples were analysed.

5.2.5 Clinical Analyses - ESR and CRP

ESR and CRP, standard clinical analyses, were determined for only the first baseline sample at Epsom General Hospital. The methods are briefly described in the appendix.

5.3 Data Analysis and Statistics

GraphPad InStat statistics package was used for all the statistical analysis, except for the correlations where NCSS97 software was used.

Comparisons between patients and controls were made using unpaired two-tailed t tests. A Welch correction or a non-parametric Mann-Whitney test was used if necessary.

A repeated measures ANOVA, followed by a Student-Newman-Keuls multiple comparison post-test, was used when comparing measurements from the same patients or control subjects at different time points during the depletion or loading protocols. Depletion samples were compared with baseline 1, with a separate ANOVA for baseline 2 and loaded samples. Two separate ANOVAs were required as the extremely large differences between depleted levels and loaded levels may mask significant differences between baseline and depleted samples. Comparison of original baseline and baseline 24 hours after depletion for the same patients or control subjects was determined using a paired two-tailed t test.

The ratios between different kynurenines were also calculated to demonstrate how much metabolite was produced from its substrate, which may indicate the activity of the enzyme catalysing the reaction. Differences between brain injury patients and controls in the ratios of QUIN: kynurenic acid and 3-hydroxykynurenine: kynurenic acid also show if the pathway has changed to favour one route more than another. In addition, the ratios are advantageous in that they indicate pathway activity, rather than actual levels of metabolites which can be quite variable in human subjects. Ratios were analysed using the same statistical tests as the metabolites themselves.

To overcome any masking of significant differences due to the natural variability between human subjects, the changes in the levels of kynurenines from baseline to depleted or loaded for individual subjects were calculated. The mean change for control subjects and patients with brain injury have then been compared at each time point using a two-tailed t test.

In all cases a significance threshold of 5% ($P<0.05$) was employed.

Correlations between tryptophan, kynurenines, ratios of kynurenines, neopterin, lipid peroxidation, ESR and CRP were assessed using a correlation matrix. Spearman correlations were used as some of the data were non-parametric.

5.4 Results

5.4.1 Kynurenines

5.4.1.1 Tryptophan

There was no difference between the baseline levels of tryptophan for control and patient samples at both baseline 1 and 2 (Figure 5-1A). At 5 hours after tryptophan depletion, there was a significantly higher level of tryptophan in the patient samples compared with the controls. Tryptophan levels were similar for patients and controls by 7 hours after depletion (Figure 5-1A). After tryptophan loading, patient tryptophan levels were significantly higher at both 5 and 7 hours after loading compared with the controls (Figure 5-1B).

The tryptophan depleting mixture, as expected, produced a major reduction of blood tryptophan concentrations at both 5 and 7 hours for controls and patients (Figure 5-1A). At the second baseline, 24 hours after tryptophan depletion, tryptophan levels had returned to levels similar to the original baseline levels for both controls and patients. Following the tryptophan load, both patients and controls showed a large increase in tryptophan levels at 5 and 7 hours. There were also significant decreases in tryptophan concentrations between 5 and 7 hours after loading for both controls and patients (both $p<0.001$).

When the changes in tryptophan levels on depletion were compared, tryptophan levels decreased to a similar extent for patients as for controls (Figure 5-2A). However, on loading, the levels of tryptophan increased more for the patients than the controls (Figure 5-2B). This suggests that when given a tryptophan load, the patients maintain a higher proportion of the tryptophan in their blood whereas in controls more of the tryptophan load is metabolised.

5.4.1.2 Kynurenine

The initial baseline kynurenine levels were significantly higher for patients than for controls and continued to be higher in patients at five hours after depletion (Figure 5-3A). At 7 hours after depletion, there was no difference between patients and controls, nor was there any difference at the second baseline. The levels of kynurenine remained similar between patients and controls after loading, suggesting that the higher levels of tryptophan in the patients is not being metabolised to kynurenine(Figure 5-3B).

As with tryptophan, the levels of kynurenine fell following depletion and increased after loading for both controls and patients. No significant difference between the original baseline sample and the second baseline sample was observed for either controls or patients. There was a decrease in kynurenine levels between 5 and 7 hours after loading, however this was only significant for the controls ($p<0.001$).

When the changes in kynurenine concentrations were examined, there was a significantly greater decrease in kynurenine levels for patients than controls at 7 hours after depletion (Figure 5-4A). Changes after loading were similar for controls and patients (Figure 5-4B).

5.4.1.3 K:T Ratio

As shown in Figure 5-5A, the K:T ratio at baseline 1 was significantly higher in patients than in control subjects and remained higher 5 hours after depletion. This suggests that the kynurenine pathway was activated in the patient group, as the K:T ratio indicates IDO/TDO activity. However, on loading, patients had a lower K:T ratio compared with controls, which was significant at 7 hours after loading, suggesting that there was more metabolism via the kynurenine pathway in the controls compared with the patients after loading. The K:T ratios after loading were significantly higher for both patients and controls.

5.4.1.4 Kynurenic Acid

Brain injury patients had a significantly lower level of the neuroprotectant KYNA in the initial baseline blood samples compared to controls, which still appeared to be lower, but not significantly, at baseline 2 (Figure 5-6A). Otherwise, KYNA levels were not significantly different between patients and controls throughout the depletion and loading protocol (Figure 5-6).

As with tryptophan and kynurenine, the levels of KYNA decreased following depletion and increased after loading for both patients and controls. There was a decrease in KYNA levels between 5 and 7 hours after loading, however this was only significant for the controls ($p<0.001$).

When the changes in KYNA were examined, there was significantly less of a decrease in KYNA for patients compared with controls following depletion at both 5 and 7 hours (Figure 5-7A), due to the lower level of baseline KYNA. The increases in KYNA on loading were not significantly different between patients and controls (Figure 5-7B).

5.4.1.5 Kynurenic Acid: Kynurenine Ratio

The KYNA: KYN ratio was significantly lower for patients at baseline 1 compared with controls suggesting that a lower proportion of the available kynurenine was being converted into KYNA in the patients by the KAT enzymes (Figure 5-8A). There was a similar trend at baseline 2 but this was not significant. However, following loading, the KYNA: KYN ratio was higher in patients, becoming significant after 7 hours (Figure 5-8B).

For controls, the KYNA: KYN ratio decreased on depletion (significant at 7 hours) and increased on loading (significant at 5 hours). The KYNA: KYN ratios for patients remained low until after loading when there was a large increase, suggesting a significant activation of KAT after loading.

5.4.1.6 3-Hydroxykynurenine

Levels of 3HKYN became undetectable during the tryptophan depletion protocol in control and patient groups, so only baseline 2 and tryptophan loaded samples were assessed. Baseline levels of 3HKYN and levels 5 hours after loading were significantly lower in the patients compared with the controls (Figure 5-9A).

Compared with baseline samples, 5 hours after tryptophan loading there was a significant increase in 3HKYN levels in patients and controls. There was a decrease in 3HKYN levels between 5 and 7 hours after loading, being significant only for the controls ($p<0.05$) (Figure 5-9A).

The change in the concentrations of 3HKYN at 5 hours after loading was significantly less for the patients than for the controls, but there was no significant difference at 7 hours after loading (Figure 5-10A).

5.4.1.7 3-Hydroxyanthranilic Acid

Levels of 3HANA also became undetectable during the tryptophan depletion protocol in control and patient groups, therefore only baseline 2 and tryptophan loaded samples were assessed. Patients had lower baseline levels of 3HANA compared with controls, which remained significantly lower at 5 hours after loading (Figure 5-9B).

At both 5 and 7 hours after tryptophan loading, the 3HANA levels were significantly increased for both the patient and control groups (Figure 5-9B), with a decrease between 5 and 7 hours ($p<0.01$ for both patients and controls).

The change in 3HANA levels due to tryptophan loading showed no significant differences between patients and controls (Figure 5-10B).

5.4.1.8 3-Hydroxykynurenine: Kynurenine ratio

The ratios of 3HKYN: KYN were significantly lower at baseline and at 5 hours after loading for patients compared with controls (Figure 5-11A), suggesting that patients produce less 3HKYN from the available kynurenine than controls.

5.4.1.9 3-Hydroxykynurenine: Kynurenic Acid ratio

As for the ratios of 3HKYN: KYN, there was a lower ratio of 3HKYN: KYNA for patients compared with controls at baseline and at 5 hours after loading (Figure 5-11B).

5.4.1.10 3-Hydroxyanthranilic Acid: 3-Hydroxykynurenine ratio

There were no significant differences between the ratios of 3HANA: 3HKYN for patients and controls at baseline or at 5 or 7 hours after loading.

5.4.1.11 Quinolinic Acid

The large variability in the QUIN concentrations for both controls and patients resulted in no significant differences between patients and controls at any time point during the depletion and loading protocols (Figure 5-12).

Levels of QUIN appeared to be decreasing slowly for both patients and controls after tryptophan depletion, with levels at their lowest at 24 hours after depletion, the time point for the second baseline (Figure 5-12A). Levels of tryptophan, kynurenine and KYNA all decreased much more quickly after depletion, with significant decreases at 5 hours after depletion, with levels of these compounds returning to baseline at 24 hours after depletion. There was a significant increase in QUIN levels at both 5 and 7 hours after loading for controls and at 7 hours for patients. QUIN concentrations remained similar between 5 and 7 hours after loading for the controls, but appeared to increase for the patients. This increase however was not significant. All the other kynurenines measured showed a decrease between 5 and 7 hours after loading. These results suggests that there is a lag time for QUIN responses to depletion and loading compared to the other kynurenines.

In an attempt to reduce the large variability in the raw QUIN data, the change in QUIN levels were examined and compared. There were still no significant differences between patients and controls (Figure 5-13).

5.4.1.12 Quinolinic Acid: 3-Hydroxyanthranilic Acid ratio

The ratio of QUIN: 3HANA appeared higher in patients compared with controls at baseline and after loading, but this was only significant at 7 hours after loading (Figure 5-14). This suggests more activity of the enzyme, 3HAO, which converts 3HANA to QUIN, for patients after loading.

5.4.1.13 Quinolinic Acid: Kynurenic Acid ratio

The ratios of QUIN: KYNA were calculated over the depletion and loading protocols. There were no significant differences between patients and controls at any of the time points, but it was noted that the initial QUIN: KYNA ratio was higher in patients than controls at the initial baseline and that on loading, the ratios were lower for patients than controls (Figure 5-15).

5.4.1.14 Neopterin

At baseline, the level of neopterin was significantly higher for patients than for controls (Figure 5-16A). At 5 and 7 hours after loading, neopterin levels were similar for patients and controls. Unexpectedly, neopterin levels in the controls at both 5 and 7 hours after loading were significantly higher than at baseline, suggesting an increase in inflammation in healthy controls after tryptophan loading. There was no change in neopterin levels after loading for the patients.

5.4.1.15 Lipid Peroxidation

Patients had significantly higher levels of lipid peroxidation products than controls at baseline and at both 5 and 7 hours after loading (Figure 5-16B). Similar to the neopterin results, the levels of lipid peroxidation increased in the controls after tryptophan loading, being significantly higher than baseline levels at both 5 and 7 hours after loading. Patient lipid peroxidation levels did not significantly increase after loading.

5.4.1.16 ESR and CRP

The ESR was significantly elevated in patients compared to controls at baseline (Figure 5-17A). The concentration of CRP in the blood of patients was significantly higher than those of controls at baseline (Figure 5-17B).

5.4.1.17 Correlations

As there were only 15 brain injury patients and 15 control subjects, fewer significant correlations were expected than if there had been larger groups. However there were many significant correlations between the kynurenines as they are all part of the dynamic kynurenine pathway. The most interesting correlations observed, which may have implications for understanding the biology of the kynurenines are discussed.

For brain injury patients at baseline 1, there was a correlation between the K:T ratio and ESR ($p<0.05$) and a negative correlation between CRP and the KYNA: KYN ratio ($p<0.01$). The K:T ratio correlated negatively with the KYNA: KYN ratio at 5 hours after depletion ($p<0.05$). The K:T ratio correlated with neopterin at baseline 2 ($p<0.05$). These results suggest activation of IDO and inhibition of KAT activity. In the patient group, neopterin correlated with QUIN ($p<0.05$) at 5 hours after loading and with kynurenine

($p<0.05$) at 7 hours after loading. In controls, the K:T ratio correlated with CRP ($p<0.05$) at baseline.

Within the brain injury patient group, at 5 hours after loading there were positive correlations for the K:T ratio with KYNA ($p<0.01$), with 3HKYN ($p<0.05$) and with QUIN ($p<0.05$). Similar correlations were also observed at 7 hours after loading (K:T ratio with KYNA $p<0.01$, with 3HKYN $p<0.05$, with QUIN $p<0.01$), but no such correlations were found for the baseline samples. For controls after loading, there were similar correlations between K:T ratio and KYNA ($p<0.001$ at 5 hours and $p<0.01$ at 7 hours) and K:T and 3HKYN ($p<0.05$ at 5 hours and $p< 0.001$ at 7 hours), but no correlation with QUIN at either time point.

For the patients and not the controls, the K:T ratio after loading also correlated with the QUIN: 3HANA ratio ($p<0.05$ at 5 hours and $p<0.01$ at 7 hours). At 7 hours after loading but not at 5 hours, QUIN correlated positively with 3HKYN ($p<0.05$) and 3HANA ($p<0.001$).

Lipid peroxidation correlated negatively with 3HANA at 5 hours after loading in patients ($p<0.05$) whereas there was a positive correlation in the controls at 5 hours ($p<0.05$).

The ages of the patients correlated with ESR ($p<0.01$) at baseline 1 and with neopterin at baseline 2 ($p<0.05$) and at 5 and 7 hours after loading ($p<0.01$ for both). In the controls, age correlated with neopterin only at 5 hours after loading ($p<0.05$).

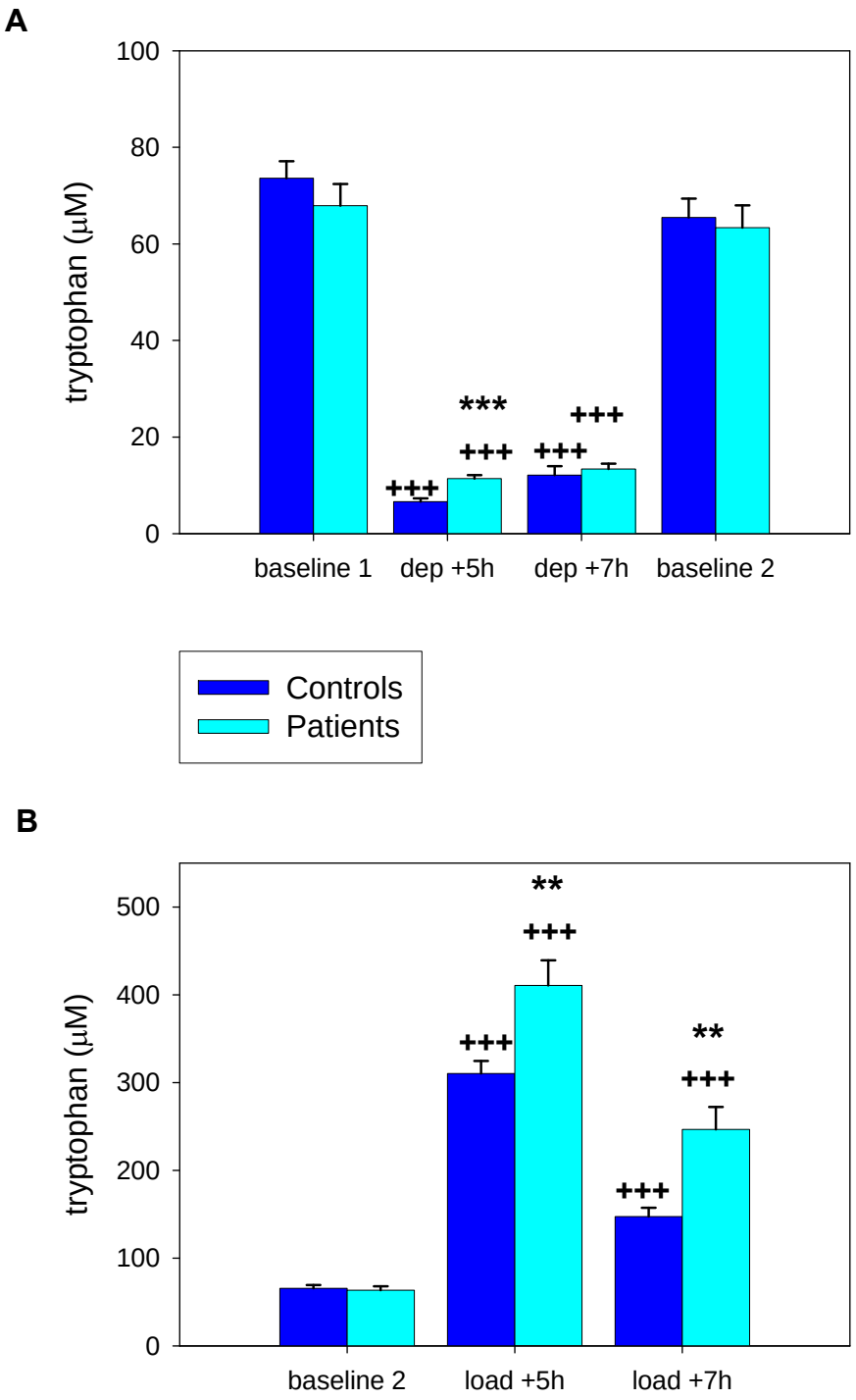


Figure 5-1 Effect of tryptophan depletion and loading on tryptophan levels in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: ** $p<0.01$, *** $p<0.001$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: +++ $p<0.001$.

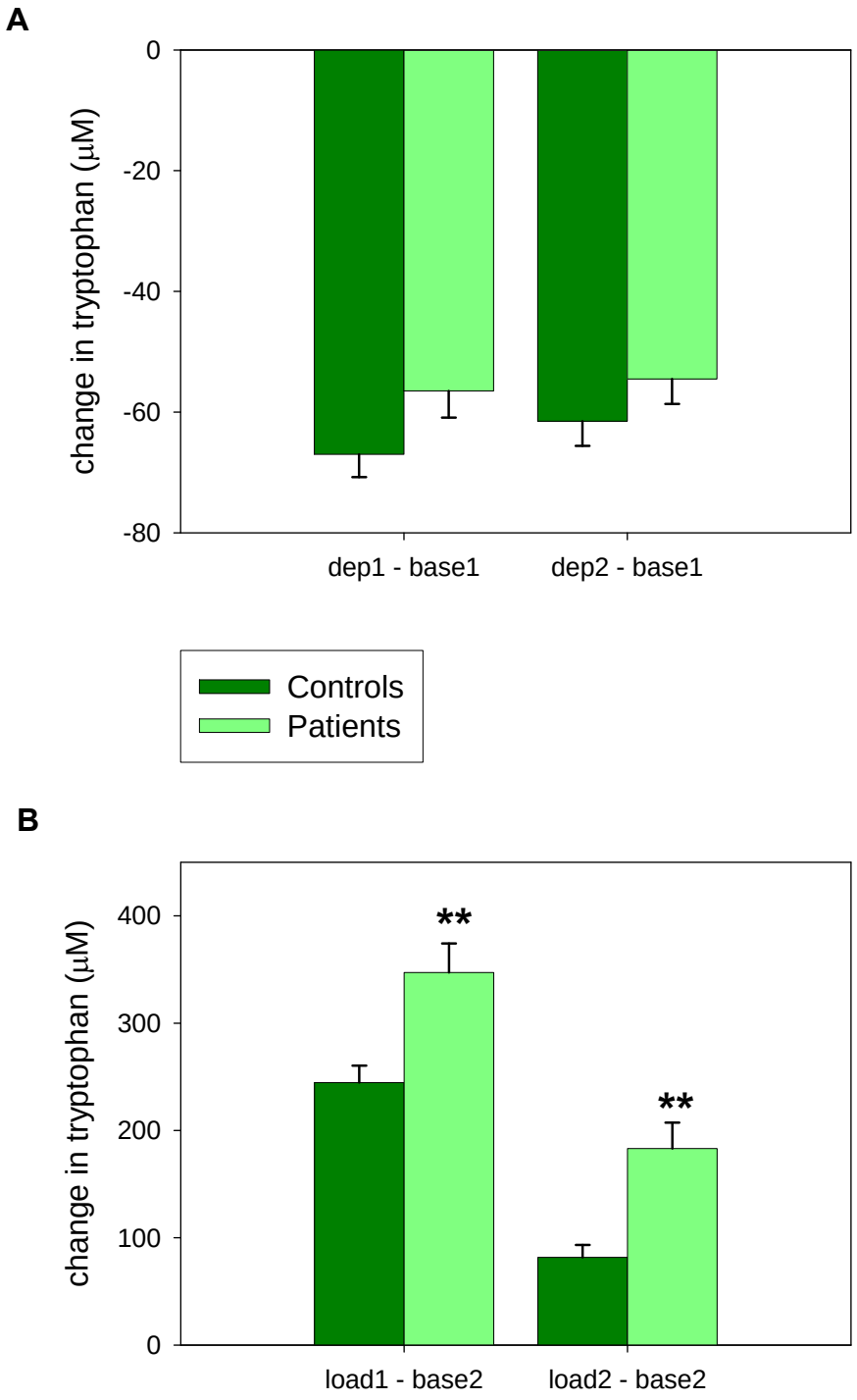


Figure 5-2 Changes in the levels of tryptophan following tryptophan depletion (A) or loading (B) for plasma from Brain Injury patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: ** $p<0.01$.

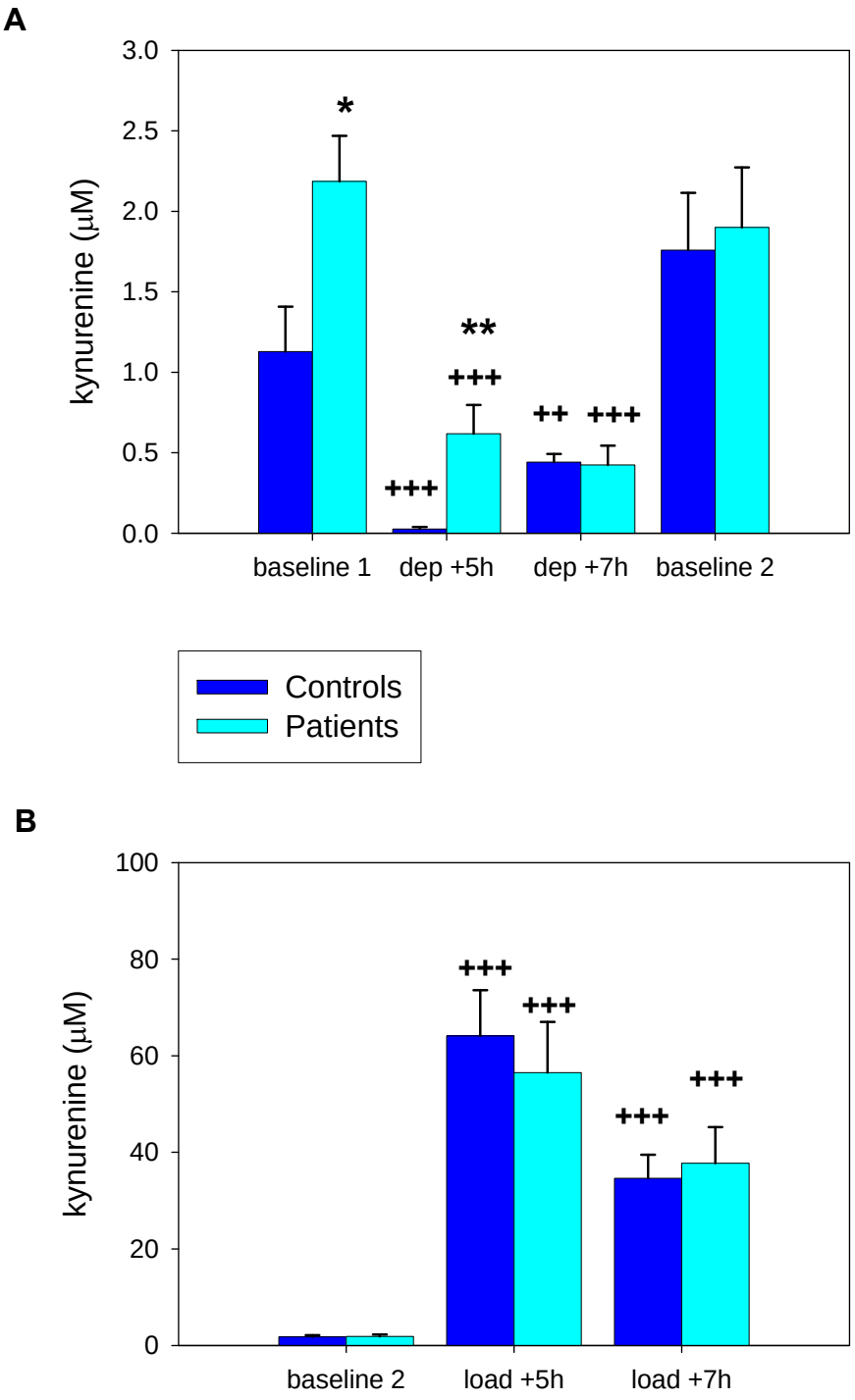


Figure 5-3 Effect of tryptophan depletion and loading on kynurenine levels in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: ++ $p<0.01$, +++ $p<0.001$.

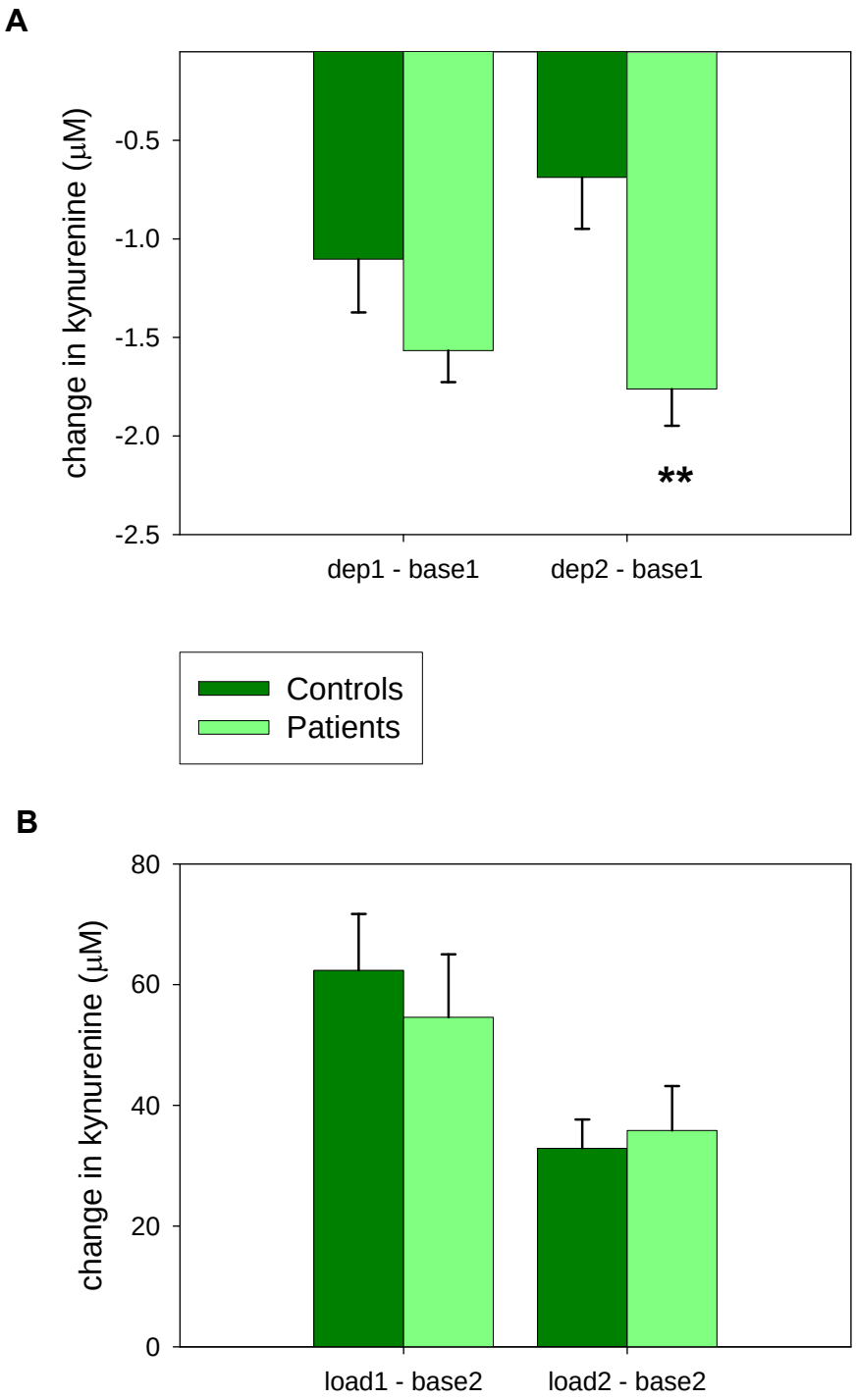


Figure 5-4 Changes in the levels of kynurenine following tryptophan depletion (A) or loading (B) for plasma from Brain Injury patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: ** $p<0.01$.

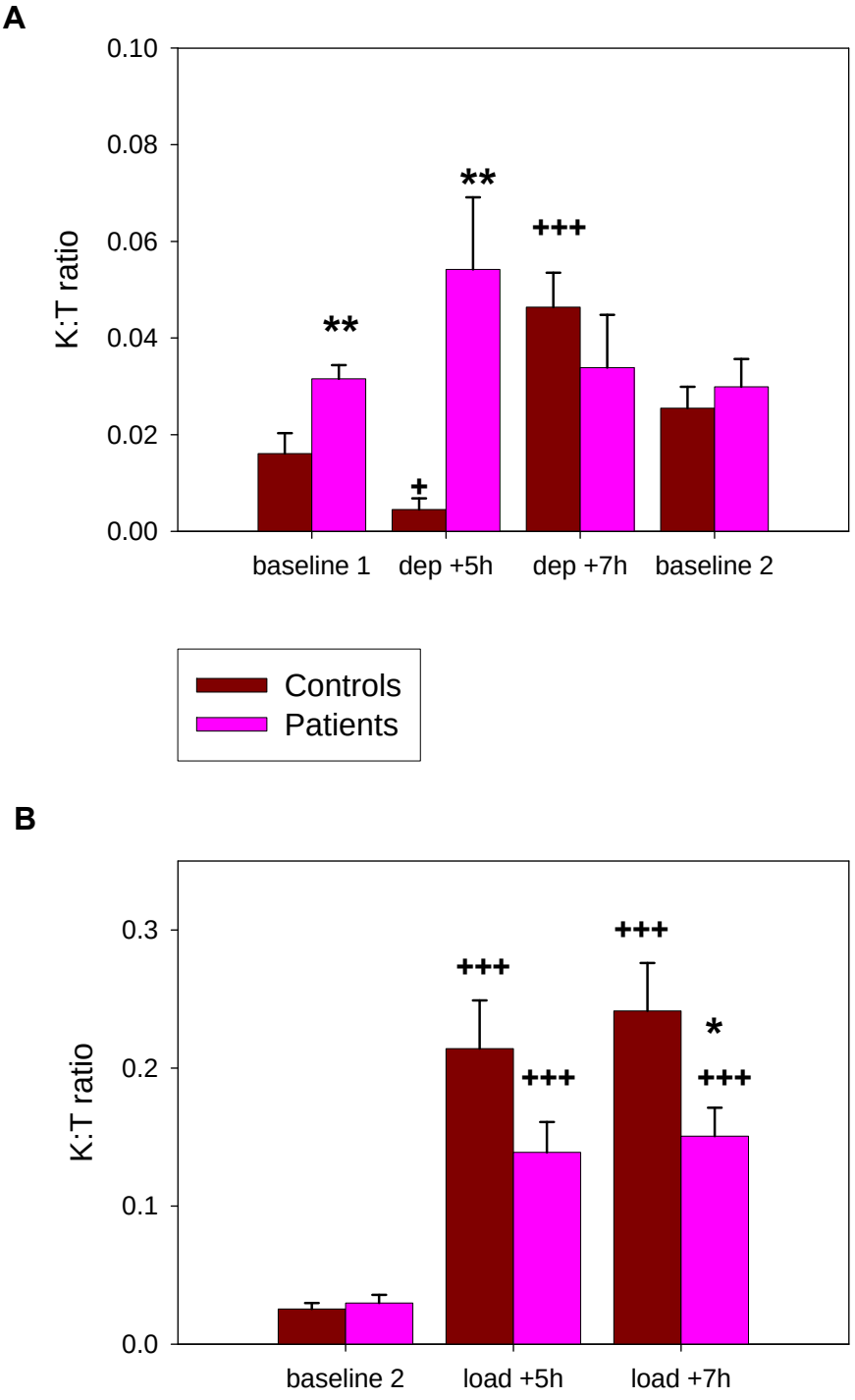


Figure 5-5 Effect of tryptophan depletion and loading on kynurenine:tryptophan (K:T) ratio in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p < 0.05$, ** $p < 0.01$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: + $p<0.05$, +++ $p<0.001$.

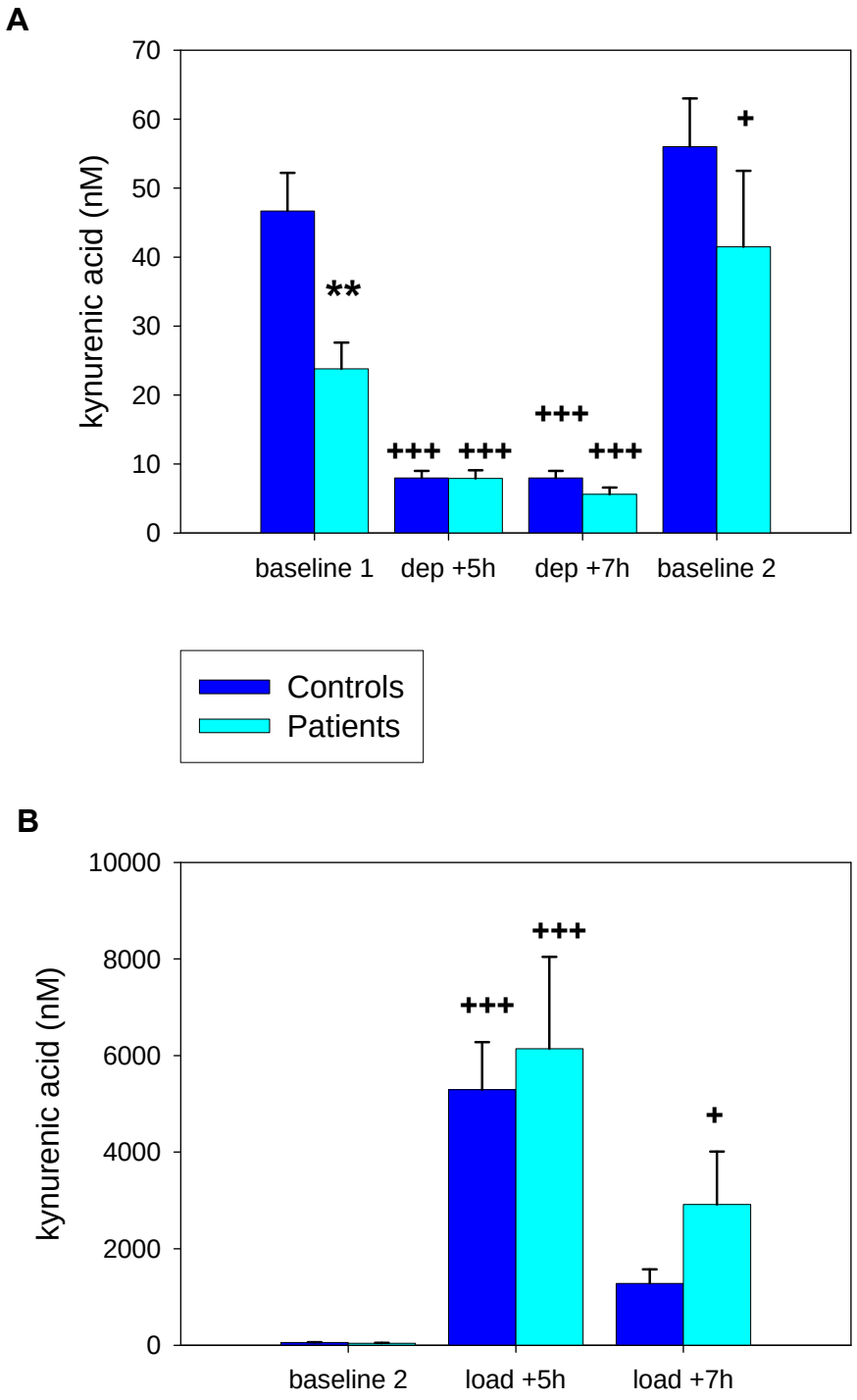


Figure 5-6 Effect of tryptophan depletion and loading on kynurenic acid levels in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: ** $p < 0.01$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: + $p<0.05$, +++ $p<0.001$.

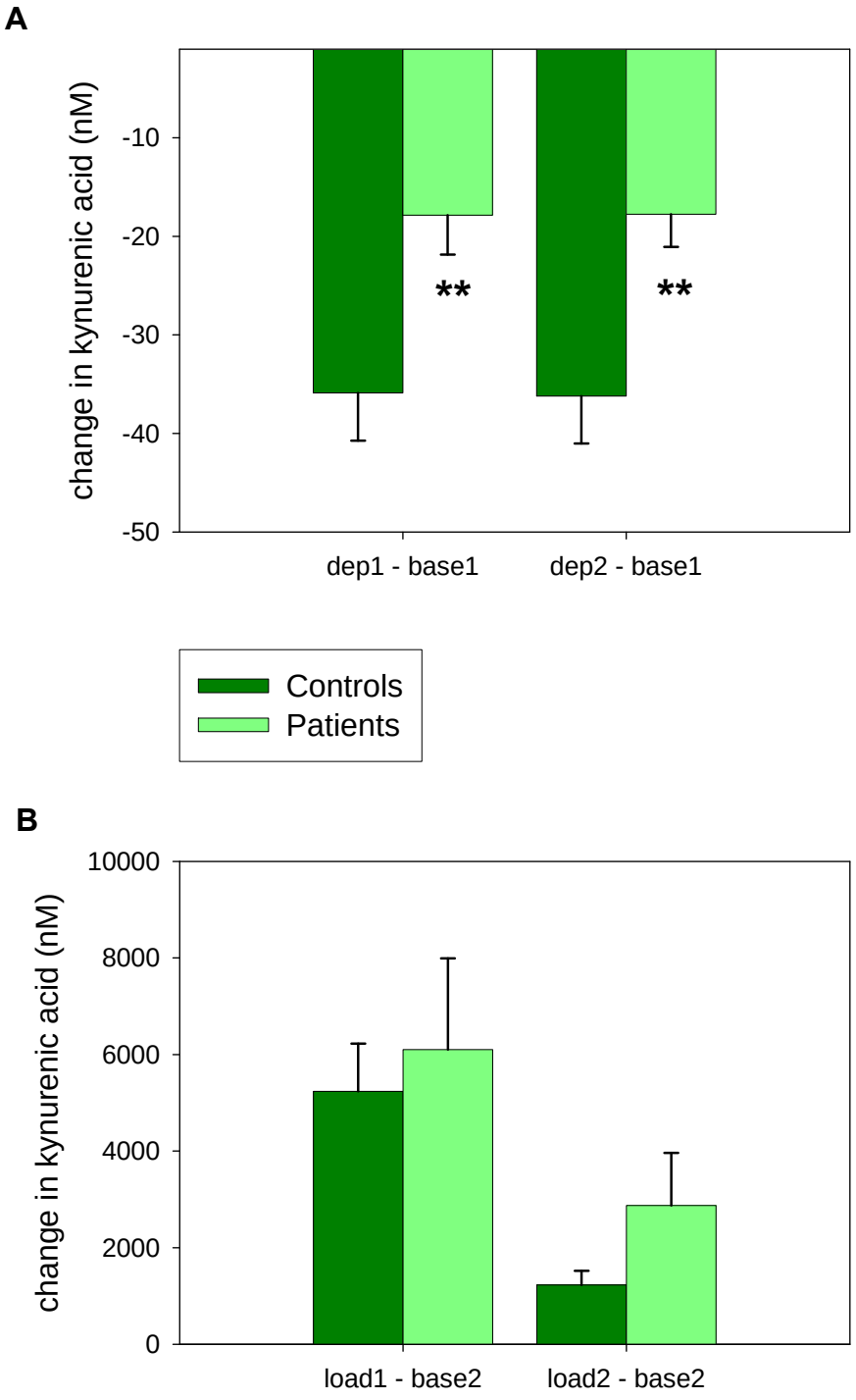


Figure 5-7 Changes in the levels of kynurenic acid following tryptophan depletion (A) or loading (B) for plasma from Brain Injury patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. Significant differences between patients and controls: ** $p<0.01$.

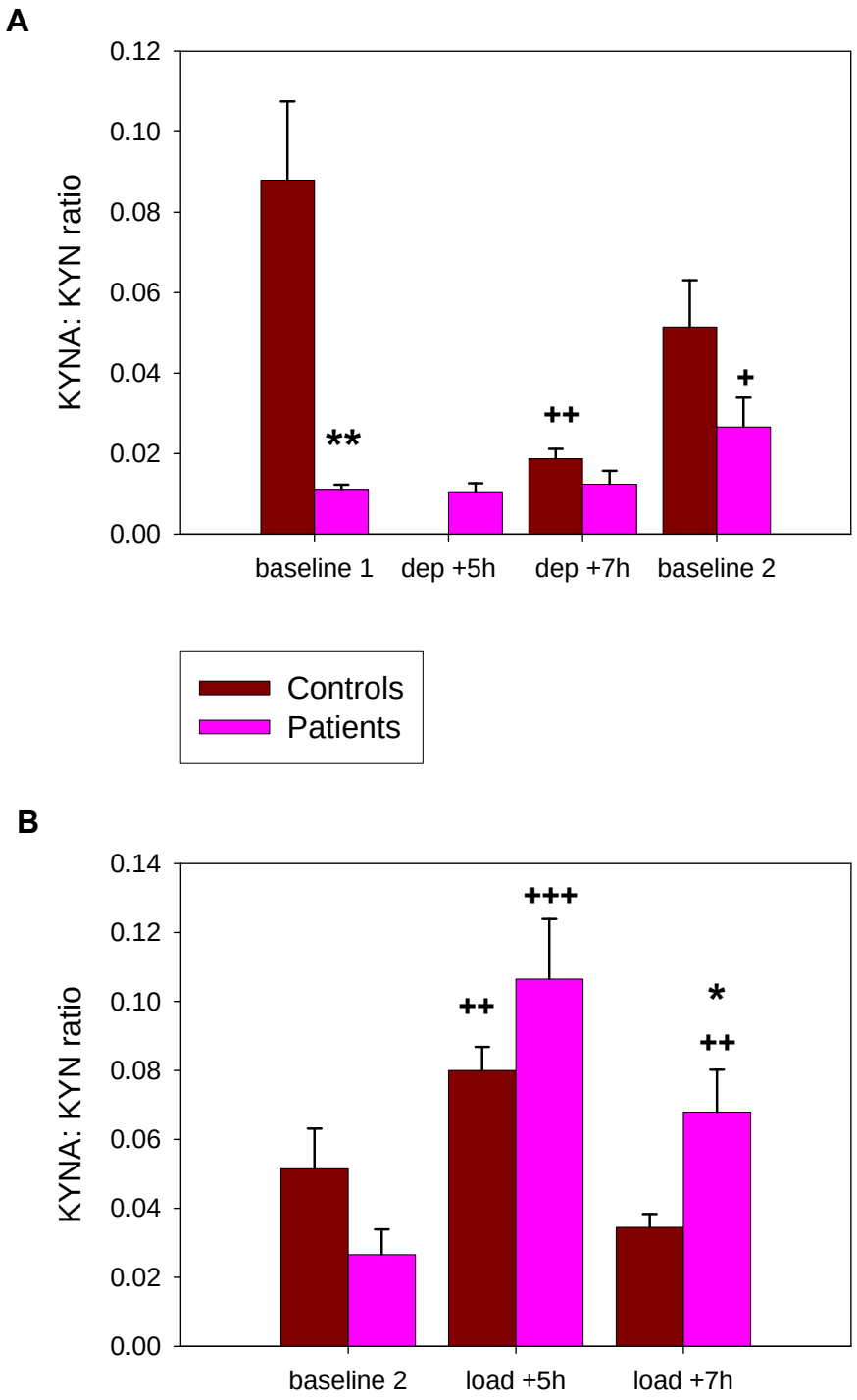


Figure 5-8 Effect of tryptophan depletion and loading on kynurenic acid: kynurenine ratio (KYNA: KYN) in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$. (Many control kynurenine levels were 0 at 5h after depletion and KYNA: KYN ratios could not be calculated, therefore were omitted from the graph).

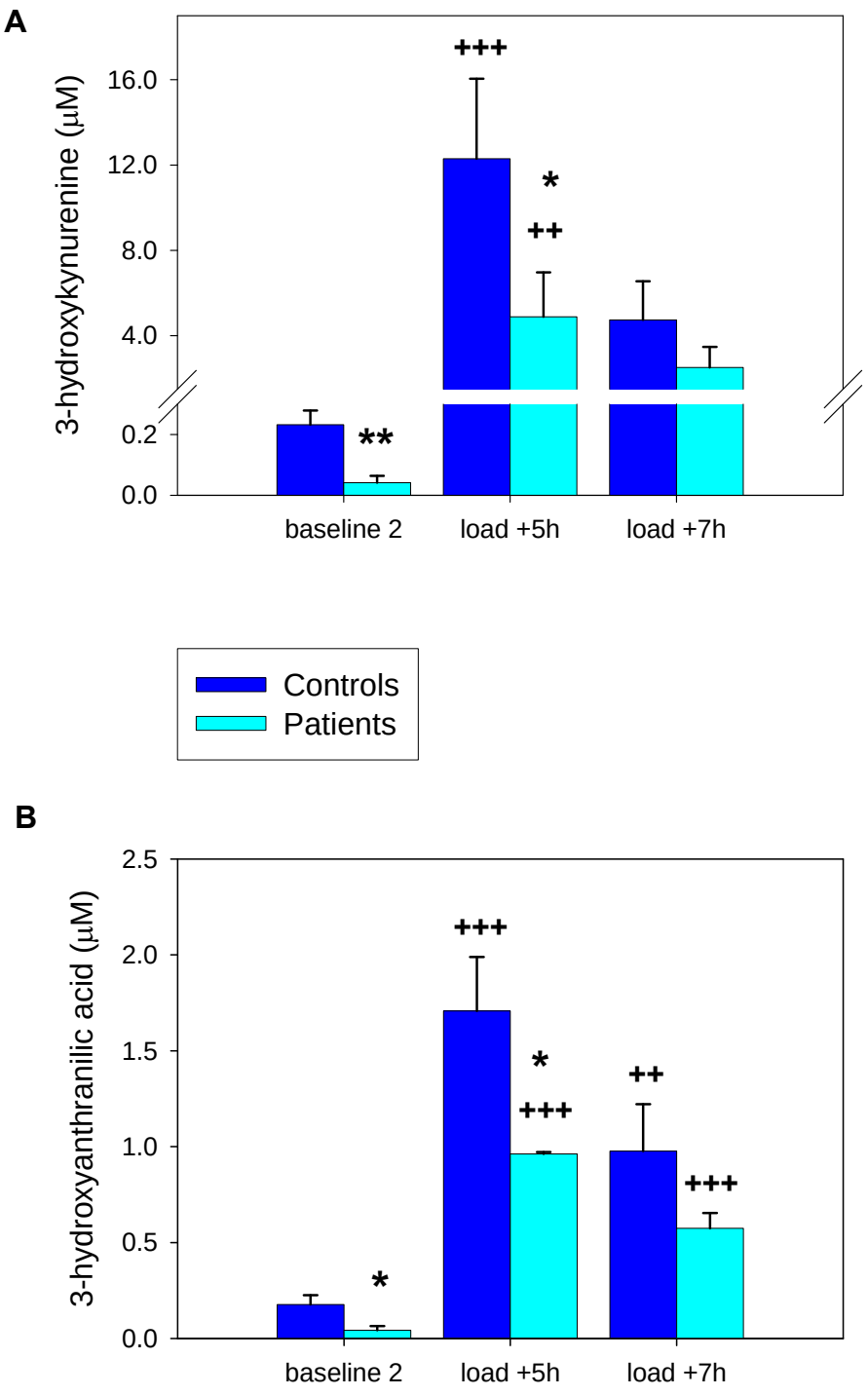


Figure 5-9 Effect of tryptophan loading on the levels of 3-hydroxykynurenine (A) and 3-hydroxyanthranilic acid (B) in the plasma of controls and Brain Injury patients at baseline 2 (24h after depletion) and at 5 and 7 hours after loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$. Effect of loading compared with baseline 2: ++ $p<0.01$, +++ $p<0.001$.

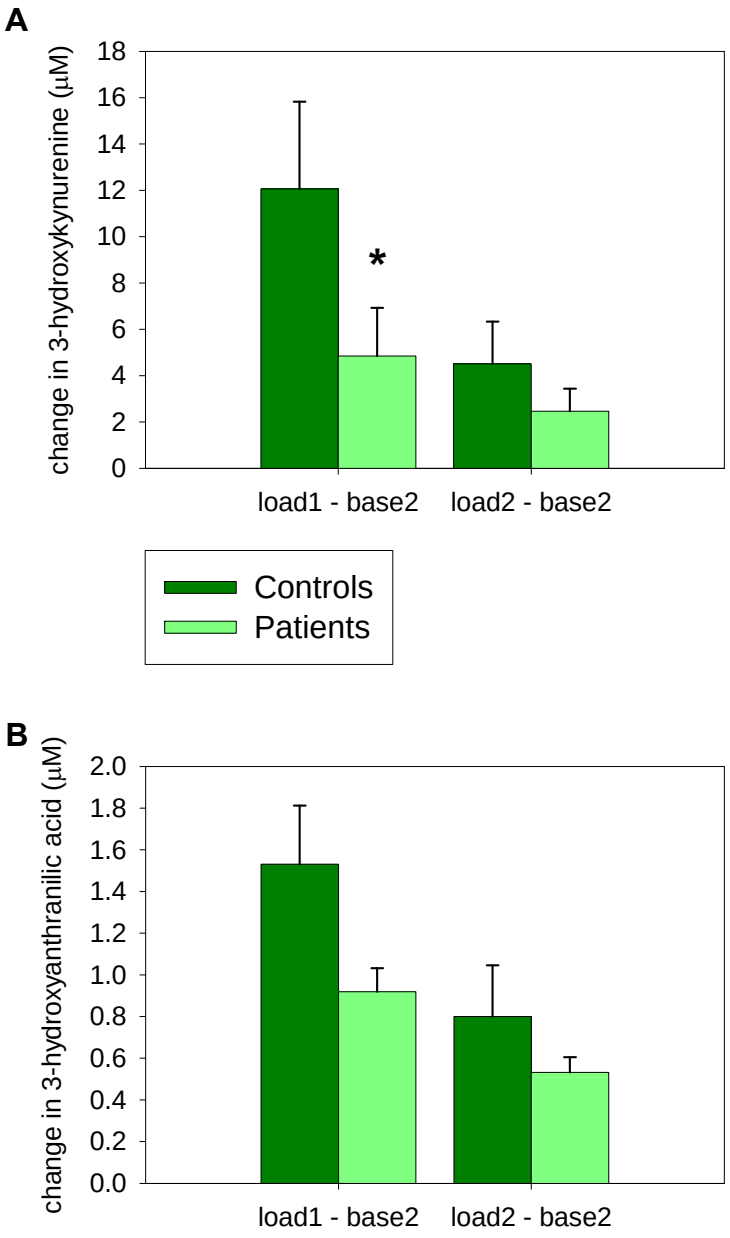


Figure 5-10 Changes in the levels of 3-hydroxykynurenine (A) and 3-hydroxyanthranilic acid (B) following tryptophan loading for plasma from Brain Injury patients and controls. Load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the loaded sample and baseline 2. Significant differences between patients and controls: * $p<0.05$.

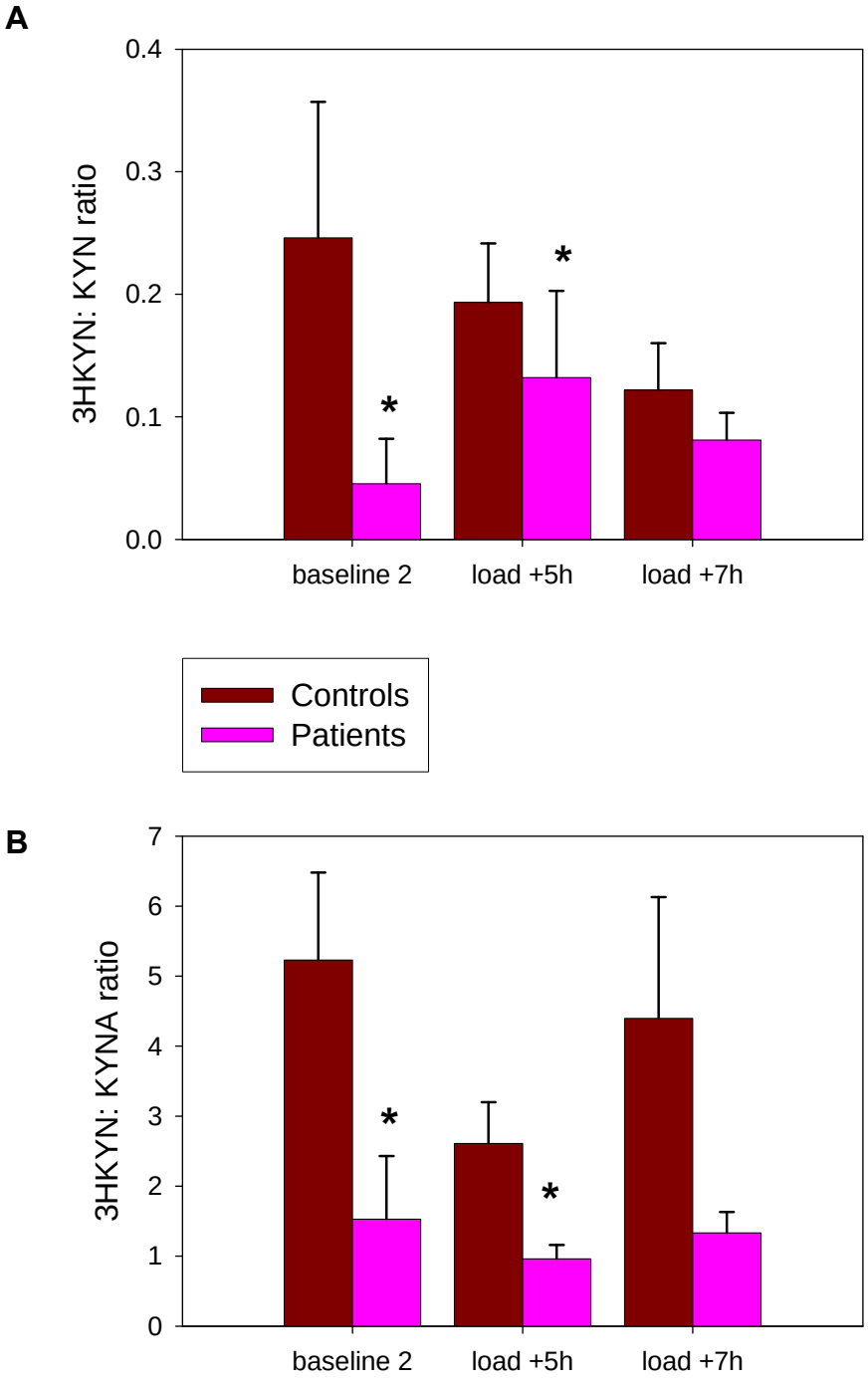


Figure 5-11 Effect of tryptophan loading on 3-hydroxykynurenine: kynurenine (3HKYN: KYN) ratio (A) and 3-hydroxykynurenine: kynurenic acid (3HKYN: KYNA) ratio (B) in the plasma of controls and Brain Injury patients. Values are mean ± SEM. Significant differences between patients and controls: * $p < 0.05$.

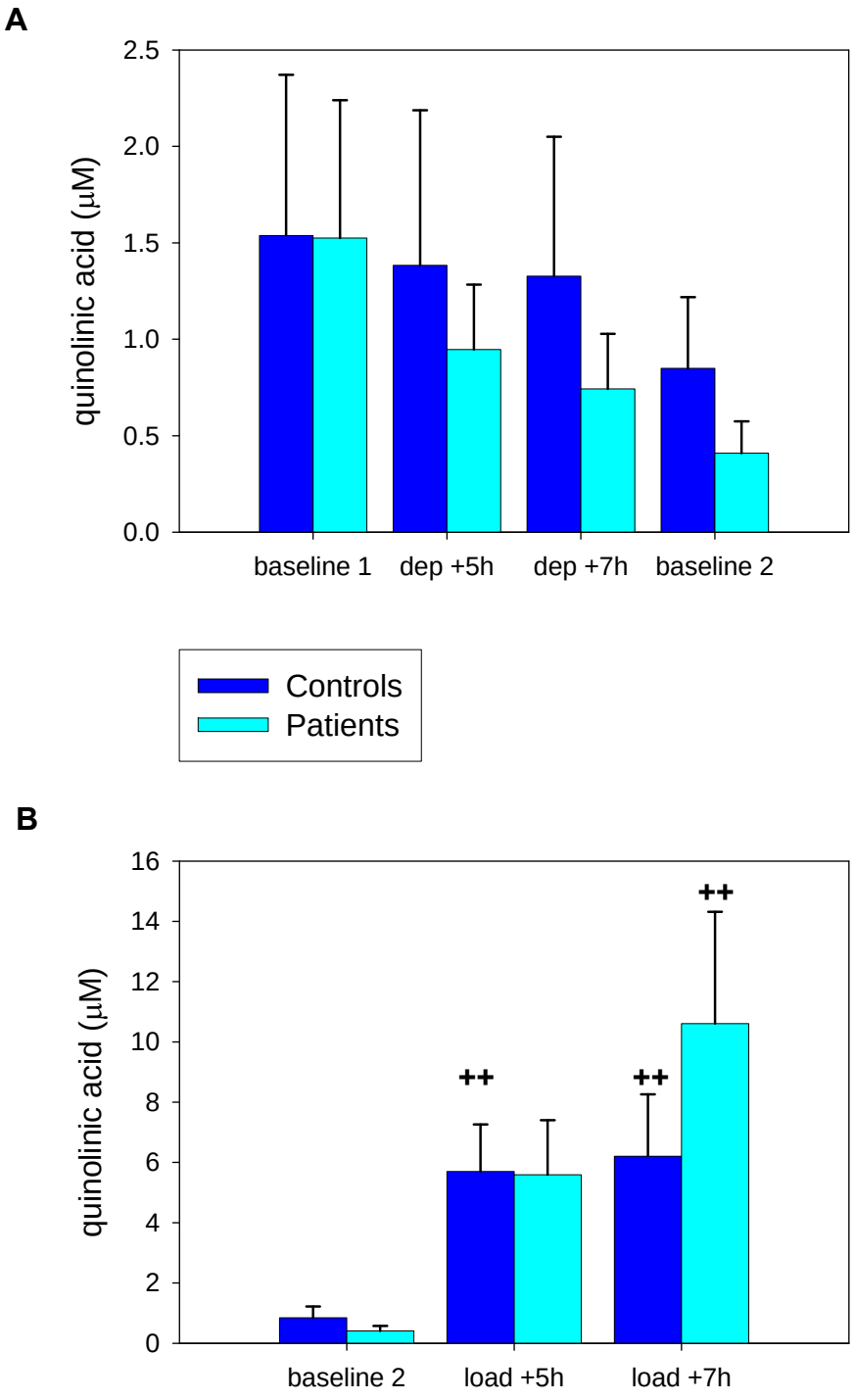


Figure 5-12 Effect of tryptophan depletion and loading on the levels of quinolinic acid in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. No significant differences between patients and controls. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: **++** $p<0.01$.

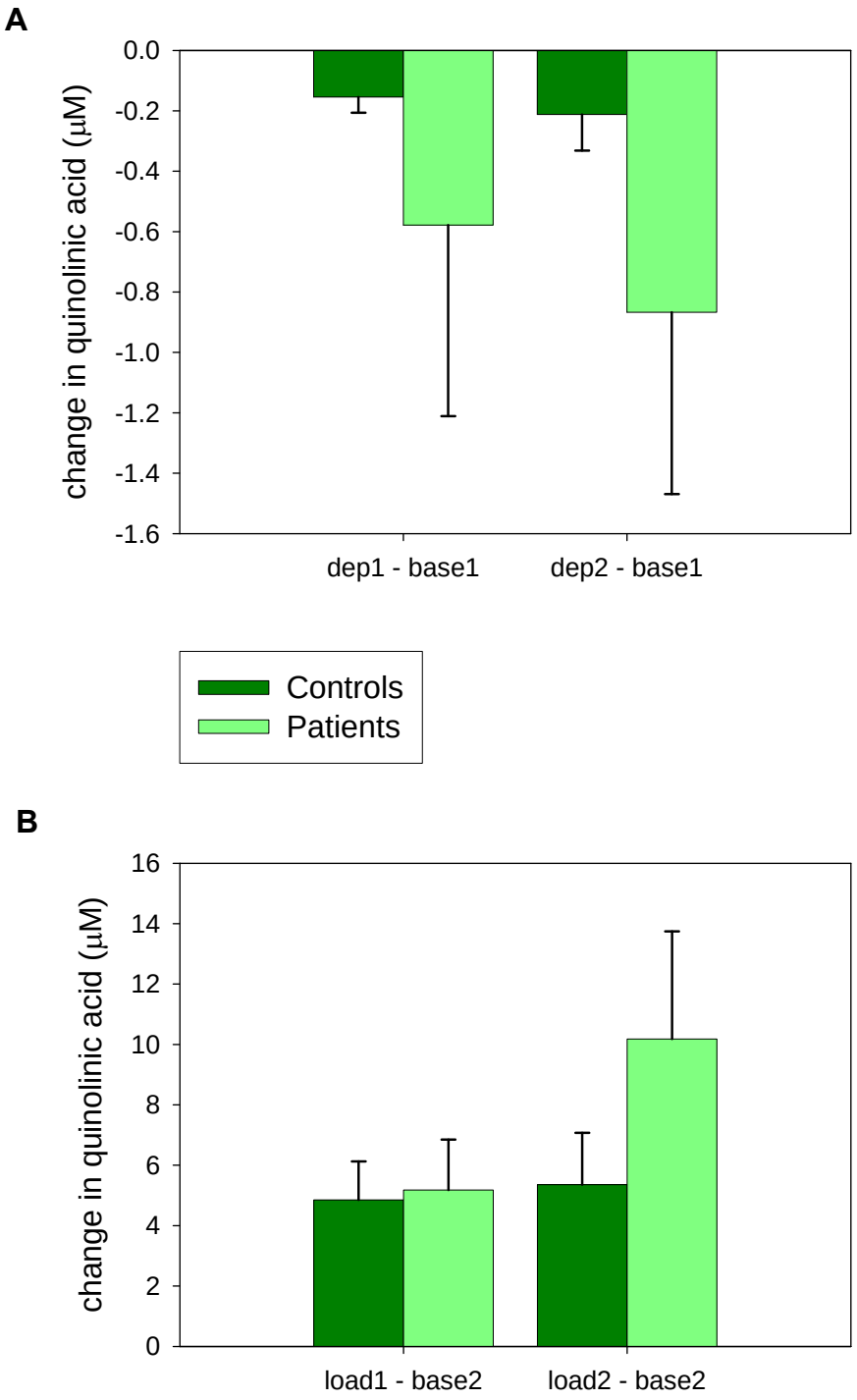


Figure 5-13 Changes in the levels of QUIN following tryptophan depletion (A) or loading (B) for plasma from Brain Injury patients and controls. Dep1 is 5 hours after depletion, dep2 is 7 hours after depletion, load1 is 5 hours after loading and load2 is 7 hours after loading. Values are mean ± SEM for differences between the depleted or loaded sample and the relevant baseline. No significant differences between patients and controls.

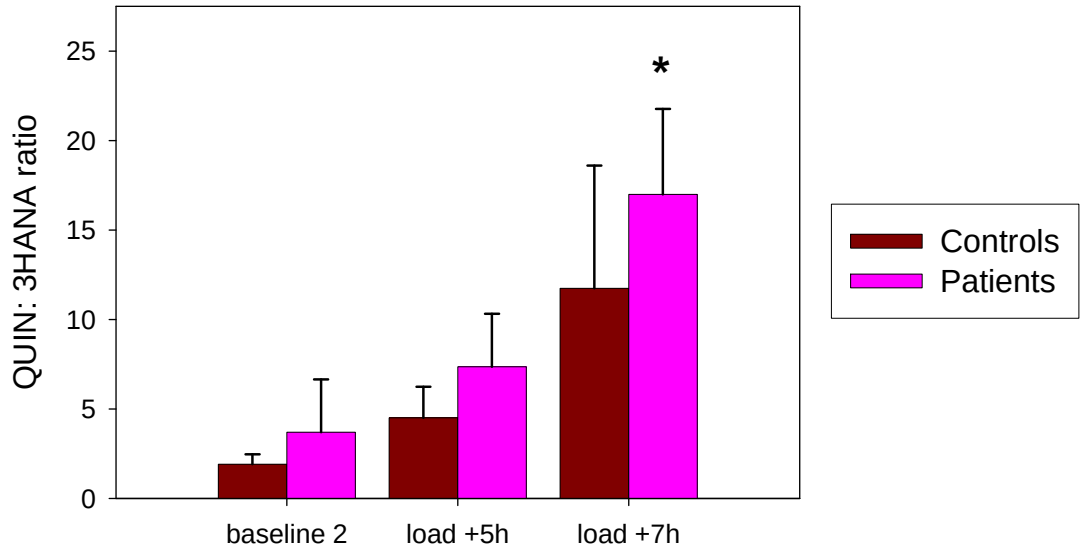


Figure 5-14 Effect of tryptophan loading on QUIN: 3-hydroxyanthranilic acid ratio (QUIN: 3HANA) in the plasma of controls and Brain Injury patients. Values are mean ± SEM. Significant differences between patients and controls: * $p < 0.05$.

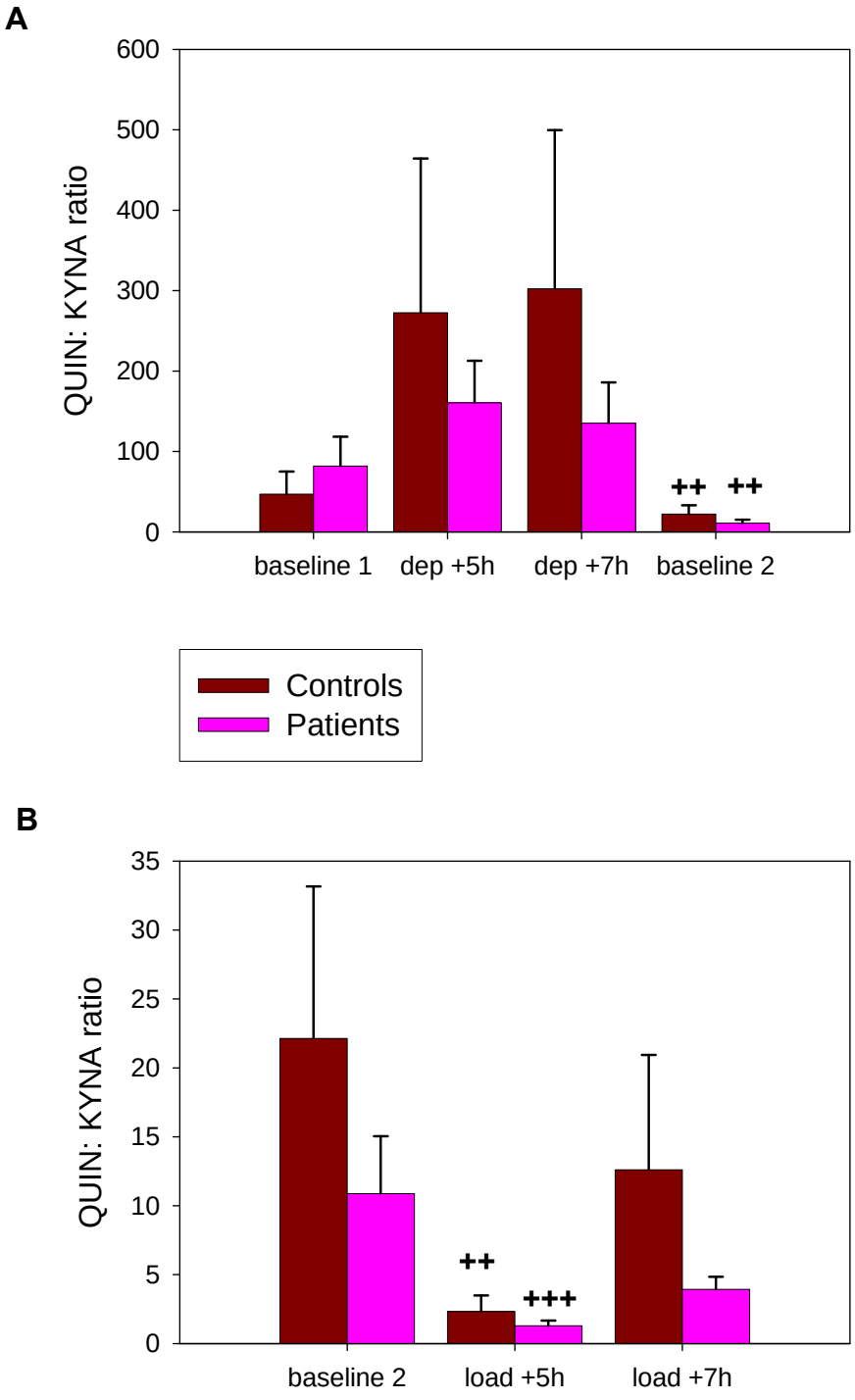


Figure 5-15 Effect of tryptophan depletion and loading on QUIN: kynurenic acid ratio (QUIN: KYNA) in the plasma of controls and Brain Injury patients, (A) at the initial baseline 1, following depletion of tryptophan and at baseline 2 (24h after depletion) and (B) at baseline 2 and following tryptophan loading. Values are mean ± SEM. No significant differences between patients and controls. Effect of depletion compared with baseline 1, effect of loading compared with baseline 2 or differences between baselines 1 and 2: **++** $p<0.01$, **+++** $p<0.001$.

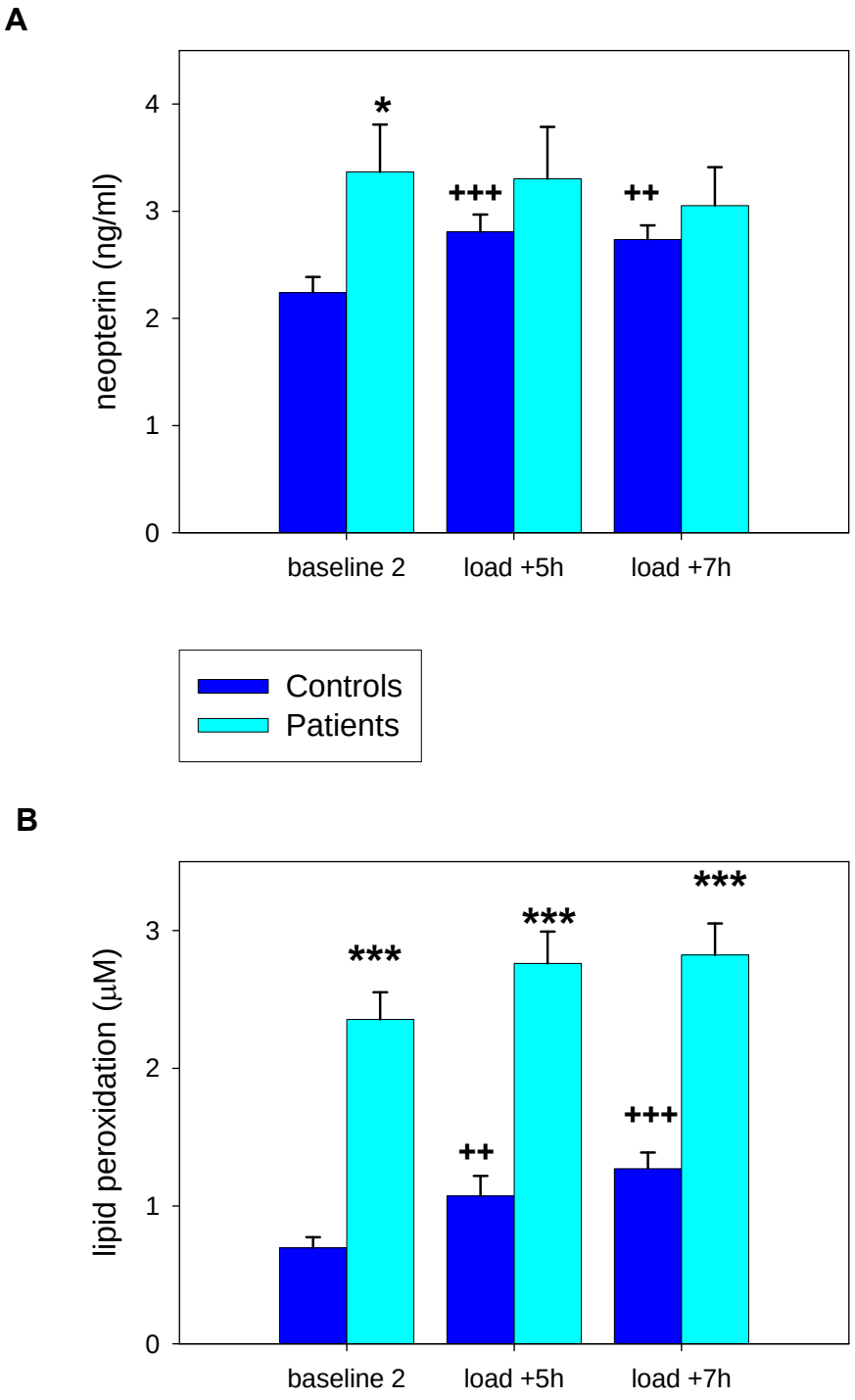


Figure 5-16 Effect of tryptophan loading on the levels of neopterin (A) and lipid peroxidation products (B) in the plasma of controls and Brain Injury patients at baseline 2 (24h after depletion) and at 5 and 7 hours after loading. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, *** $p<0.001$. Effect of loading compared with baseline 2: ++ $p<0.01$, +++ $p<0.001$.

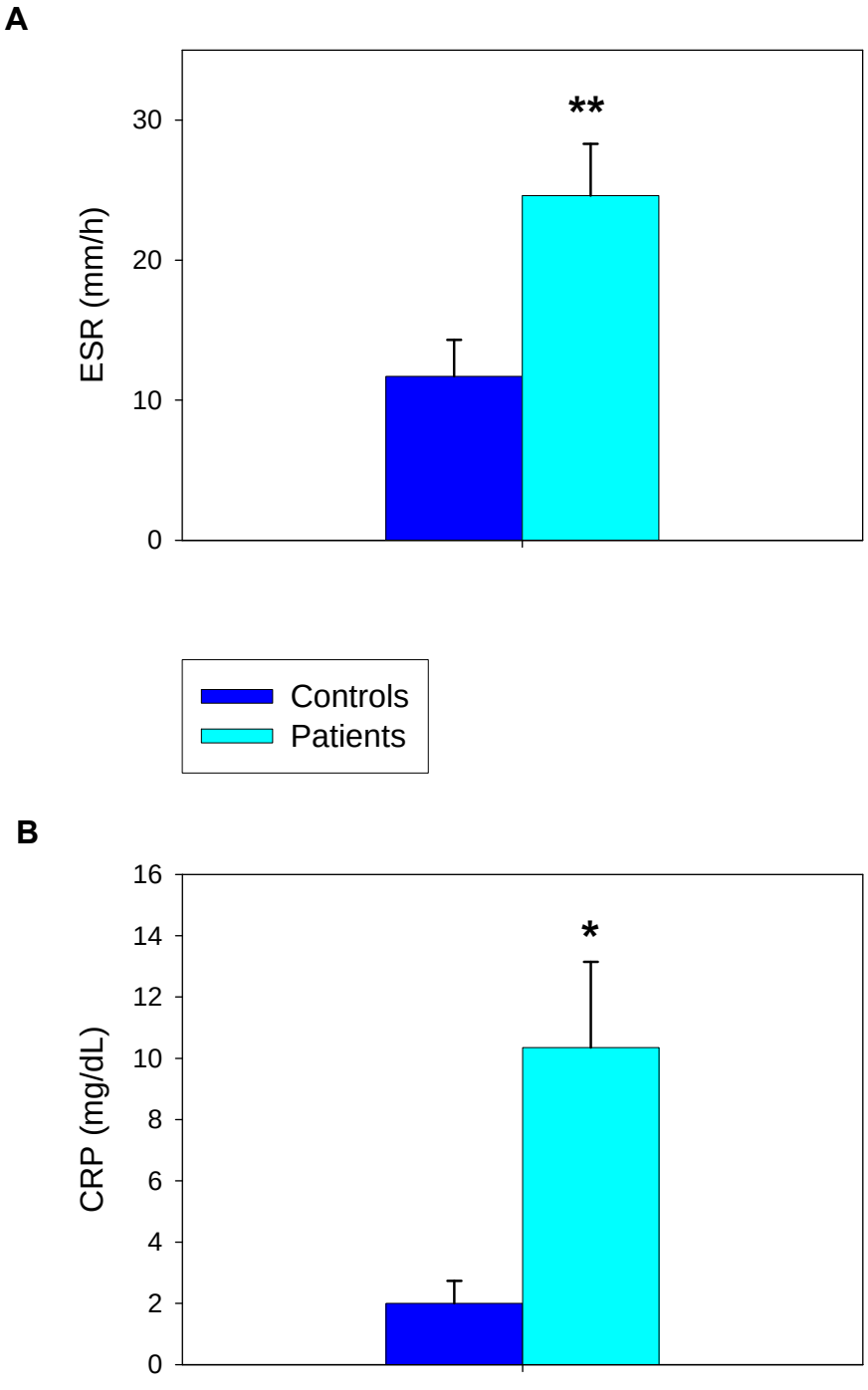


Figure 5-17 Comparison of ESR and CRP between Brain Injury patients and controls at baseline. Values are mean ± SEM. Significant differences: * $p<0.05$, ** $p<0.01$.

5.5 Discussion

5.5.1 Tryptophan Depletion and Loading

The depletion and loading protocols produced similar effects in the brain injury patients as they did for the HD patients, with QUIN levels in the blood taking longer to respond to the depletion or loading than the other kynurenines. It was interesting that QUIN levels appeared to be higher in brain injury patients than in controls at 7 hours after loading, as they did in HD patients, but in neither case were these increases significant due to the large variability in the QUIN results. In retrospect, it would have been useful to measure the QUIN levels in the blood at later time points, but no further blood samples had been taken. Even without an inflammatory stimulus, tryptophan loading activates the kynurenine pathway in controls, suggesting increased activity of TDO in the liver. Indeed, tryptophan has been shown to activate TDO (Schutz *et al*., 1972). Tryptophan loading for the patients showed increased metabolism through all the branches of the pathway, as the K:T ratio correlated with KYNA, 3HKYN and QUIN. For controls after loading, there were similar correlations between K:T and 3HKYN and KYNA but interestingly no correlations with QUIN, suggesting restricted production of the neurotoxin, QUIN, in healthy controls.

5.5.2 Tryptophan, Kynurenine and IDO/TDO Activity

There was no difference between the levels of baseline tryptophan in patients and controls, in agreement with Aquilani *et al*. (2003), where the patient group had been in hospital for two months after severe TBI. In our study, the higher level of tryptophan at 5 hours after depletion and the larger changes in tryptophan on loading for the patients suggest that the brain injury patients used less tryptophan for other metabolic processes (such as protein synthesis) than the controls.

The higher K:T ratios for patients at baseline and at 5 hours after tryptophan depletion suggest increased activity of IDO, which occurs in conjunction with increased levels of the inflammatory marker, neopterin. Patients with increased elevation in their inflammatory status demonstrated greater activation of the pathway, as the patient group showed significant correlations between the K:T ratio and ESR at baseline 1 and between the K:T ratio and neopterin at baseline 2. Elevated K:T ratios have also been associated with a reduction in cognitive performance of patients with Alzheimer's disease (Widner *et al*., 2000).

However, at 7 hours after loading, there was a lower K:T ratio in the patients compared with the controls, which was due to the patients' higher tryptophan levels. As kynurenine levels were similar after loading, this may suggest the tryptophan was at such a high level in both patients and controls that IDO/TDO was working at its maximum rate for both groups. There was still a relationship between inflammatory status and increased activation of the pathway for the patients, as there were correlations between neopterin and both kynurenine and QUIN after loading.

5.5.3 Kynurenic Acid and KAT Activity

For patients with chronic brain injury, the lower level of the neuroprotectant KYNA in their blood may have contributed to the brain damage in these patients and may be a factor in preventing recovery or causing further brain damage. The importance of KYNA was demonstrated in two studies using a rat model of TBI, as KYNA treatment dramatically reduced the cognitive dysfunction (Smith *et al*., 1993) and reduced the neuronal damage in the hippocampus (Hicks *et al*., 1994).

Together with the lower levels of KYNA, there was also a significantly lower KYNA: KYN ratio for patients at the initial baseline, suggesting low KAT activity with little conversion of kynurenine to KYNA compared with controls. However, after loading, the KYNA: KYN ratio was higher in patients than controls, becoming significant at 7 hours after loading, suggesting that with a tryptophan load, KAT becomes more activated in patients than in controls. The changes in KAT activity in patients throughout the depletion and loading protocols were the opposite to those of IDO/TDO activity. At baseline and 5h after depletion, the increased kynurenine present in patients and, hence, the increased K:T ratios, may be partly due to very low activity of KAT. Indeed, there was a negative correlation between the K:T ratio and the KYNA: KYN ratio at 5 hours after depletion for patients. The KYNA: KYN ratio also correlated negatively with CRP. As the K:T ratio correlated with the inflammatory markers, neopterin and ESR at baselines, increased inflammation together with increased pathway activity is linked with a decrease in the production of KYNA from kynurenine. The lower K:T ratio in patients with tryptophan loading may indeed be partly a reflection of the higher KYNA: KYN ratio, as more kynurenine was converted to KYNA. It is interesting that significant effects in both ratios are seen only at 7 hours following the tryptophan load, showing that differences in enzyme activities, between patients and controls, are becoming more pronounced at 7 hours. These results suggest that for patients with chronic brain injury, a diet high in tryptophan would

stimulate production of the neuroprotectant, KYNA, and may have a contribution in limiting further brain damage.

5.5.4 3-Hydroxykynurenine and 3-Hydroxyanthranilic acid and Oxidative Stress

Increased levels of 3HKYN have been shown in the brains of HD patients, but not in Alzheimer's disease brains (Pearson and Reynolds, 1992). Both 3HKYN and 3HANA can generate reactive oxygen species such as hydrogen peroxide, causing oxidative stress, resulting in neuronal damage and apoptosis (Eastman and Guilarte, 1990, Okuda *et al.*, 1996). However, in this study the chronic brain injury patients showed lower levels of both 3HKYN and 3HANA than controls at baseline and 5 hours after loading, although levels of lipid peroxidation products were higher. In addition, the ratios of 3HKYN: KYN and 3HKYN: KYNA were both lower for patients at baseline and at 5 hours after loading, suggesting that for patients, less 3HKYN was being produced from kynurenine and that there was even less 3HKYN being produced compared to the other branch of the pathway producing KYNA, where activity was also lower for patients.

Both 3HKYN and 3HANA could have autoxidised, resulting in the formation of reactive oxygen species (Dykens *et al*., 1987; Ishii *et al*., 1992; Hiraku *et al*., 1995), which may have contributed to the increased oxidative stress. The low levels of 3HKYN and 3HANA may suggest that these metabolites have been metabolised more quickly than they were produced.

Alternatively, the low levels of 3HKYN and 3HANA in the patient group may be a protective compensatory response to the presence of a high level of reactive oxygen species. Another explanation may be that as 3HKYN and 3HANA are redox-active, they can act as antioxidants, which is dependent on the presence of other redox-active compounds, such as transition metal ions (Christen *et al*., 1990; Leipnitz *et al.*, 2007). The lower levels of 3HKYN and 3HANA present in the blood of brain injury patients would then suggest that there was less antioxidant activity to inhibit the oxidative stress. These hypotheses suggest that mechanisms other than the direct generation of reactive oxygen species from the kynurenine pathway metabolites may cause oxidative stress in chronically brain injured patients. Such other mechanisms may include macrophages and microglia releasing free radicals or activation of NMDA receptors with calcium influx into the cells, resulting in the production of reactive oxygen species (Sahuquillo *et al*., 2001).

5.5.5 Quinolinic Acid

For TBI, Sinz *et al*. (1998) reported a large increase in QUIN in the CSF in humans. Following transient cerebral ischaemia in the gerbil, increases in IDO activity and QUIN levels were measured in the brain but blood QUIN and L-kynurenine levels remained unchanged (Saito *et al*., 1993b). In our study, no significant differences in QUIN between patients and controls were observed throughout, although there was a trend showing that QUIN may be accumulating in patients after loading. Indeed, the ratio of QUIN: 3HANA was significantly higher at 7 hours after loading in the patients, showing increased metabolism of QUIN from its substrate, 3HANA. It may have been expected that there would be lower levels of QUIN produced after loading for the patients, as there were significantly lower levels of its precursors, 3HKYN and 3HANA, at baseline 2 and 5 hours after loading. This may have been overcome by increased activity of 3HAO, as shown by the trend for increased QUIN: 3HANA ratios in patients. Although there was no increase in QUIN levels for the patients, the QUIN: KYNA ratio was slightly higher for patients than controls at the initial baseline, although this was not significant, suggesting there may have been a slight shift towards QUIN production in the patients.

The changes in the levels of QUIN in response to tryptophan depletion and loading appear to occur over a longer timescale than for the other kynurenines. This can account for the significantly lower ratio of QUIN: KYNA at baseline 2 and at 5 hours after loading for both patients and controls. KYNA but not QUIN levels have returned to baseline levels following depletion by baseline 2 and KYNA levels but not QUIN are close to maximum at 5 hours after loading. This suggests that QUIN formation occurs more slowly than the other kynurenines, which may be associated with the activity of the enzyme, 3HAO, which synthesises QUIN.

After loading, the K:T ratio correlated with QUIN and the QUIN: 3HANA ratio, suggesting that patients with higher activation of the pathway also produced higher levels of QUIN. Higher K:T ratios are associated with a higher inflammatory status and interestingly neopterin also correlated with QUIN at 5 hours after loading for the patients. These effects were observed after loading, which may be expected as loading produced increased levels of all the kynurenine metabolites. As these correlations were not observed for the controls, these results are consistent with the view that inflammation induces the kynurenine pathway which produces the neurotoxin, QUIN.

5.5.6 Inflammation: Neopterin, ESR and CRP

Levels of neopterin, ESR and CRP were significantly increased at baseline in patients with brain injury compared with controls, demonstrating persistent inflammation in these patients several years after the brain injury. During the first week after TBI, Lenzlinger *et al*. (2001) showed an increase in serum neopterin concentrations to a maximum of approx. 4.5ng/ml. After maintaining this high neopterin level for 1 week, patients showed a decrease in the levels of neopterin during the third week to just above 3ng/ml, although by the end of the third week neopterin levels were still slightly elevated in patients compared with their upper normal limit (2.5ng/ml). However, for CSF, once increased, high neopterin levels were maintained throughout the second 2 weeks of the study. It is interesting that in our study with patients who had sustained the brain injury at least 1 year previously, the mean serum neopterin level was still at 3.3ng/ml for patients compared with 2.2ng/ml in controls at baseline. Inflammation is often associated with oxidative stress but no correlations between neopterin and lipid peroxidation were observed.

5.5.7 Lipid Peroxidation and Oxidative Stress

The levels of lipid peroxidation products, malondialdehyde and 4-hydroxynonenal, continued to be elevated for the patients with chronic brain injury, where the injury was sustained at least one year prior to this study. In contrast, early onset lipid peroxidation after severe human TBI has been demonstrated in the CSF (Cristofori *et al*., 2001). The levels of the lipid peroxidation product, malondialdehyde, were high for patients on admission (on average 3 hours after trauma), but by 48 hours levels had reduced considerably but were still higher than in controls. CSF samples from infants and children who had suffered severe TBI showed increased markers of oxidative stress at maximum levels at day 1 and a reduced total antioxidant reserve maximal on the last time point at 5-7 days (Bayir *et al*., 2002). Pratico *et al*. (2002) demonstrated that moderate experimental TBI induced widespread brain lipid peroxidation, which was accompanied by a similar increase in lipid peroxidation in urine and plasma.

5.5.8 Conclusions

Patients with severe chronic brain injury, sustained between 1 and 35 years prior to this study, showed the presence of inflammation and an activated kynurenine pathway. The correlations show that increases in inflammatory indicators (neopterin, ESR and CRP) were linked to increases in kynurenine pathway metabolites for the brain injury patients

and not for the controls, consistent with the view that activation of the kynurenine pathway is a consequence of inflammation.

Activation of the kynurenine pathway, determined by the increased K:T ratio, was demonstrated at the initial baseline and at 5 hours after depletion. Following the depletion protocol, the second baseline sample showed no significant increase in the K:T ratio for the brain injury patients. The lower KYNA level at the initial baseline together with low KYNA: KYN ratios indicates low activity of KAT. However, after loading, the patients showed lower K:T ratios and increased KAT activity, suggesting that tryptophan loading has a neuroprotective effect. The trend for accumulating QUIN after loading indicates that the neuroprotective benefit of increased KAT activity may be outweighed by the production of the neurotoxin, QUIN.

The levels of 3HKYN and 3HANA were significantly lower in patients with chronic brain injury than in controls. These effects were continued after loading and were similar to those shown with the HD patients. These metabolites are redox-active and therefore are likely to influence the oxidative stress in these patients, especially at their elevated levels after loading. The levels of lipid peroxidation were much higher in patients compared with controls, but there were no significant increases in lipid peroxidation after loading.

6 Stroke

6.1 Introduction

Many patients who experience a stroke suffer consequent brain damage. One factor which may contribute to brain damage is the production of neurotoxic kynurenine metabolites, such as the NMDA receptor agonist, QUIN. The involvement of NMDA receptors in stroke is well established as glutamate activation of these receptors results in excitotoxicity, a major event for initiating ischaemic cell death (Dirnagl *et al*., 1999), and many NMDA receptor antagonists have shown neuroprotection in animal models of ischaemia (McCulloch, 1992; Chen *et al*., 1993; Pellegrini-Giampietro *et al*., 1994). Enhanced NMDA receptor activity may result from the activation of the kynurenine pathway, which has been suggested as a secondary inflammatory response to tissue damage in stroke (Heyes and Nowak, 1990), with the levels of kynurenines expected to rise in the initial days after the stroke and then plateau or decrease in the following weeks. There is evidence for kynurenine pathway activity in ischaemia, as increased levels of IDO activity and QUIN concentrations were observed following transient global ischaemia in gerbils (Saito *et al*., 1993b). In addition, neuroprotection in animal models of ischaemia has been demonstrated by increases in the brain levels of KYNA, with decreases in 3HKYN, 3HANA and QUIN concentrations, when KMO inhibitors were administered (Cozzi *et al*., 1999). These observations suggest that neurotoxic kynurenines in stroke have the potential to induce permanent damage in the penumbra.

This investigation was undertaken to determine if a stroke causes changes in the kynurenine pathway. If the kynurenines are altered, there is the potential for therapies to be developed to interfere with the pathway which could lead to reducing brain damage after a stroke. Blood samples were examined, which may reflect the status of the kynurenines in the brain. As a stroke usually affects one main area of the brain, the kynurenines are likely to be higher in this area of damage, and therefore changes in the peripheral levels of kynurenines may only be observed to a lesser extent. The use of blood samples, as opposed to post-mortem brains, enabled the levels of the kynurenines to be monitored for the same patients at different time points after the stroke.

In this study, the levels of kynurenine pathway metabolites in the days immediately following a stroke, and then after 7 and 14 days were examined. Tryptophan and kynurenine concentrations were determined to demonstrate activation of the kynurenine

pathway. Levels of KYNA, ANA and 3HANA were also determined to examine changes in different branches of the pathway. These metabolites were measured in the serum of stroke patients and healthy controls. In addition, to assess the state of inflammation in these patients, neopterin concentration and ESR were determined. Lipid peroxidation products, malondialdehyde and 4-hydroxynonenal, were also measured as markers of oxidative stress.

6.2 Methods

6.2.1 Patients and Samples

Fifty patients (23 males, 27 females, ages ranging from 54 to 98 (mean ± SEM, 80 ± 1.6)) who were diagnosed as having suffered a stroke were recruited from Epsom General Hospital, together with thirty five healthy subjects (11 males, 24 females, ages ranging from 46 to 81 (mean ± SEM, 67 ± 1.7)) to act as controls. Patients were recruited after obtaining ethical permission from the Ethical Committee of the Epsom and St Helier University Hospitals NHS Trust. Informed written consent was obtained from all subjects or from their next of kin.

Once patients had been recruited for the study, blood samples were taken as soon as possible. Usually the first blood sample was taken one day after the patient had the stroke, but for some patients the first blood sample was delayed by a day or two, while the patient was being recruited. For patients, samples taken 1, 2, 3, 4, 7 and 14 days after the stroke were assessed. For control samples, bloods were collected at three time points over a two week period (initial sample, after 7 days and after 14 days). After the blood had been allowed to clot, serum samples were prepared by centrifugation at 1000g for 10 minutes, and frozen within two hours of venesection. Samples were kept frozen while being transported from Epsom General Hospital to the University of Glasgow and then stored at -40ºC prior to analysis.

Patients had a computed tomography (CT) head scan, a few days after the stroke which determined whether the stroke was ischaemic or haemorrhagic. The severity of the stroke varied between patients but no established severity scale was used for these patients and therefore the most useful way to define severity was to group the patients according to whether they died during the study or immediately afterwards (within 3 weeks of the stroke), remained in hospital for the duration of the study, or were discharged from hospital

within the 14 days of the study. Data were assessed for differences between ischaemic and haemorrhagic stroke and also for differences depending on the severity of the stroke.

Some patients were given aspirin (75mg daily), which may interfere with the kynurenine pathway and therefore this subgroup of patients were compared with others not taking aspirin. A number of patients were also taking glucocorticoids and antidepressants which may interfere with the kynurenine pathway. Again, these subgroups of patients were compared with patients not taking these drugs. Many patients were also taking drugs which were considered to have minimal effect on the kynurenine pathway: anticoagulants (30%), antibiotics (42%), statins (22%) and drugs to reduce blood pressure (β-blockers, ACE inhibitors, diuretics) (48%). Patients also taking paracetamol as required, asthma inhalers, laxatives, drugs for nausea and insomnia were noted.

6.2.2 Kynurenines by HPLC

Samples of serum were extracted following the method described in Section 3.1.2. Ms L Oxford helped with some of the extractions. Tryptophan, kynurenine and KYNA were measured by HPLC using the zinc acetate mobile phase, with absorbance and fluorescence detection (Section 3.2.3). 3HANA and ANA were determined by fluorescence detection following HPLC using the sodium acetate buffer (pH 5.5) as the mobile phase (Section 3.3.3).

6.2.3 Neopterin, Lipid Peroxidation and ESR

Serum samples were analysed using the methods for determining neopterin and the lipid peroxidation products, malondialdehyde and 4-hydroxyalkenals described in the appendix and were carried out by Dr CM Forrest and Ms L Oxford at the University of Glasgow. The ESR was measured at Epsom General Hospital, for patients on admission and for ten controls for comparison at day 1. The method outline is described in the appendix.

6.2.4 CT scans

The CT scans for the stroke patients were carried out at Epsom General Hospital by Dr CD George, Consultant Radiologist, to determine whether the stroke was ischaemic or haemorrhagic. Wherever possible, the scan was performed between 4-6 days after the stroke, as this is the optimal time for an infarct to be observed on the scan. Scans were not conducted on 5 patients, who were too ill for the scan to be performed.

Dr George estimated the total volume (ml) of the damaged brain from the CT scans. This was calculated from the infarct dimensions. If a patient had two acute infarcts, the volumes of each were calculated and summed to give a total volume. With the haemorrhagic patients, the total volume for the bleed was estimated.

6.2.5 Data Analysis and Statistics

GraphPad InStat statistics package was used for all the statistical analysis, except for the correlations where NCSS97 software was used.

As well as assessing the concentrations of kynurenine metabolites in the blood, the ratios between different kynurenines were also calculated. The K:T ratio and the ANA: KYN ratio demonstrate how much metabolite was produced from its substrate, which indicate the activity of the enzymes catalysing these reactions. The ratio of 3HANA: ANA may indicate the extent of direct hydroxylation of ANA, although 3HANA is usually produced from 3HKYN. In addition, the ratios are advantageous in that they indicate pathway activity, rather than actual levels of metabolites which can be quite variable in human subjects. Ratios were analysed using the same statistical tests as the metabolites themselves.

Comparisons between patients and controls were made using unpaired two-tailed t tests at 1, 7 and 14 days after the stroke. A Welch correction or a non-parametric Mann-Whitney test was used if necessary. The sample numbers (n) for controls and patients at each day of the study are shown in Table 6-1. One-way ANOVA, followed by a Student-Newman-Keuls multiple comparison post test, was used when comparing measurements from patients on different days during the study. When required, a Kruskal-Wallis nonparametric ANOVA with a Dunn's multiple comparison post test was used. The data were also tested for linear trends occurring with time after the stroke. (Ideally a repeated measures ANOVA would have been performed but, since several patients did not start the study until day 2 or day 3 and several died during the study, this reduced the n numbers considerably. In addition, there were also some samples missing at various time points as bloods could not always be collected as patients had gone home or were too ill for blood to be taken. One-way ANOVA was chosen as the most appropriate, as much of the data was not repeated measures at the same time points.) For 32 of the control patients, there were blood samples at 1, 7 and 14 days, and therefore repeated measures ANOVA was used to assess variability in the controls.

For ESR, patients (data available for 28 patients only) were compared with ten controls using an unpaired two-tailed t test.

For both patients and controls, correlations between the concentrations of all the kynurenines, neopterin levels, lipid peroxidation measurements, ESR, subject age and CT scan lesion volume were made at each time point. Spearman correlations were used as many sets of data were nonparametric.

Ischaemic and haemorrhagic patients were compared using unpaired two-tailed t tests at 1, 2, 3, 4, 7 and 14 days after the stroke. A Welch correction or a nonparametric Mann-Whitney test was used if necessary. There were 36 patients who had suffered an ischaemic stroke and 9 a haemorrhagic stroke. The sample numbers at each day of the study for ischaemic and haemorrhagic patients are shown in Table 6-1. At days 1, 7 and 14, the ischaemic and haemorrhagic subgroups were compared with controls, using an ANOVA, followed by a Student-Newman-Keuls multiple comparison post test. When required, a Kruskal-Wallis nonparametric ANOVA with a Dunn's multiple comparison test was used. To check if either subgroup showed any effect over time, ANOVA, followed by a Student-Newman-Keuls multiple comparison post test was used for parametric data or if nonparametric, Kruskal-Wallis ANOVA with a Dunn's multiple comparison test was used. Correlations between CT scan lesion volumes for the ischaemic patients only were compared to correlations for the complete set of patients.

Similar comparisons were made when the data was grouped according to severity of the stroke. 12 patients died during or immediately after the study (within 3 weeks of the stroke), 12 were discharged from hospital during the study and 26 remained in hospital. The n numbers for these subgroups of patients are shown in Table 6-1. The same comparisons were made again when ischaemic patients were subgrouped into those taking aspirin throughout the study (n=14) and those not taking aspirin (n=11). The individual sample numbers at each day of the study are shown in Table 6-2. For severity and aspirin effects, day 14 (and day 4 for aspirin) results were not assessed as the sample numbers were very small.

Patients taking glucocorticoids (n=5) were compared with those not on glucocorticoids. Similarly, patients taking antidepressants (n=6) were compared to the other patients. Comparisons were made using unpaired two-tailed t tests at 1, 2, 3, 4, 7 and 14 days after the stroke, when the sample number was at least 3. The sample numbers for patients taking these drugs at each day of the study are shown in Table 6-2.

In all cases a significance threshold of 5% ($p<0.05$) was employed.

Table 6-1 Sample numbers at each day of the study for patients and controls, when subgrouped into ischaemic and haemorrhagic patients and when subgrouped for severity into those who died within 3 weeks of the stroke, patients who remained in hospital and those who were able to go home during the 2 week study. Not applicable as not enough data for analysis: na.

			Patient Subgroups		Patient Subgroups		
	Controls	All Patients	Ischaemic	Haemorrhagic	Died	Stayed in Hospital	Home
TOTAL	35	50	36	9	12	26	12
DAY 1	35	32	25	4	8	15	9
DAY 2	na	40	30	7	9	19	12
DAY 3	na	43	31	9	10	23	10
DAY 4	na	16	10	4	5	8	3
DAY 7	32	30	23	5	8	17	5
DAY14	34	24	17	5	na	na	na

Table 6-2 Sample numbers at each day of the study for ischaemic patients taking aspirin or not throughout the study and for patients taking other drugs. Not applicable as not enough data for analysis: na.

	Ischaemic Patients Only		Patient Subgroups taking other drugs	
	Aspirin	No Aspirin	Glucocorticoids	Antidepressants
TOTAL	14	11	5	6
DAY 1	11	6	na	4
DAY 2	14	8	5	6
DAY 3	11	10	5	6
DAY 4	na	na	3	na
DAY 7	8	7	5	5
DAY14	na	na	na	3

6.3 Results

6.3.1 Patients and Controls

6.3.1.1 Kynurenines

Tryptophan levels were significantly lower in patients compared with controls at 1, 7 and 14 days after the stroke (Figure 6-1A). Kynurenine levels were higher in patients compared with controls but these were only significant at day 1, 1 day after the stroke (Figure 6-2A). As expected from these results, the K:T ratio was significantly higher for patients than for controls at day 1, 7 and 14 (Figure 6-1B), indicating activation of the kynurenine pathway following a stroke. There was no significant difference between patients and controls for KYNA (Figure 6-2B). The ratios of KYNA: KYN were calculated, but were not significantly different from controls. Levels of ANA were significantly higher for patients compared with controls at days 1 and 7, but this difference was not significant at day 14 (Figure 6-3A). When the ratios of ANA: KYN were calculated, these were higher in patients compared with controls, but only significant at day 1. This suggests that more ANA is being produced from kynurenine immediately after the stroke than normally occurs in healthy subjects. 3HANA concentrations were significantly lower for patients compared with controls at days 1, 7 and 14, with $p<0.001$ at all three time points clearly indicating a change in 3HANA levels (Figure 6-4A). The ratio of 3HANA: ANA (Figure 6-4B) was significantly lower for patients compared with controls at each time point (days 1, 7 and 14).

There were no significant changes in the levels of tryptophan, kynurenine, KYNA, 3HANA and ANA during the 14 days following the stroke, nor were there any linear trends over time. Controls samples did not show any significant variability between day 1, day 7 and day 14 for any of the kynurenines.

6.3.1.2 Lipid Peroxidation and Neopterin

Lipid peroxidation was higher in patients' samples than in controls at day 1, day 7 and day 14 (Figure 6-5A). Although ANOVA did not show any significant differences in lipid peroxidation over the 14 days following the stroke, there was a significant linear trend ($p<0.05$), indicating that lipid peroxidation was decreasing with time after the stroke. Levels of neopterin were significantly higher for patients compared with controls at days 1,

7 and 14 (Figure 6-5B). There was no evidence of neopterin levels falling over the 14 days after the stroke, with levels remaining elevated.

For controls, there was no significant variability in neopterin between days 1, 7 and 14. However, lipid peroxidation at day 7 was significantly higher than lipid peroxidation at day 1 and at day 14. This may be due to the error bars at each time point being very small. This result is of little importance for the study as the lipid peroxidation results for the controls at day 7 were still much lower than for the patients (Figure 6-5A).

6.3.1.3 ESR

On admission to hospital, patients had a significantly higher ESR (26.23 ± 4.45 mm/h, n=28) compared with controls (12.10 ± 4.09 mm/h, n=10) ($p<0.05$).

6.3.1.4 CT Scans

The estimated total volume of brain damage varied from 0 to 158ml, with a mean volume of 26.3ml (n=40). For ischaemic patients the lesion volume varied from 0 to 135ml, with a mean of 25.6ml (n=34) and for haemorrhagic patients the lesion volume was between 1 and 158ml, with a mean volume of 30.3ml (n=6). Some patients had very small areas of infarction which were too small to be seen on the CT scan and were attributed a volume of 0 for comparison with the other volumes.

6.3.1.5 Correlations

The significant correlations between the estimated total volume of brain damage from the CT scans and the kynurenines, neopterin, lipid peroxidation and ESR are shown in Table 6-3. The volume of brain damage was greater when the levels of 3HANA in the blood were lower, correlations being significant at 3 of the 6 time points. When brain damage was correlated with 3HANA: ANA ratio, the correlations were significant at 4 time points. Significant correlations were also observed with lipid peroxidation at 2 time points.

For stroke patients many correlations were observed between the kynurenines as expected, as they are all metabolites of the dynamic kynurenine pathway (Table 6-4). Increased levels of kynurenine produced increases in both KYNA and ANA, as kynurenine correlated with KYNA and ANA at days 1, 2, 3, 7 and 14. The K:T ratio also correlated with KYNA (at days 1, 2, 3 and 7) and ANA (at days 1, 3 and 7), demonstrating that

increased activation of the pathway increases the levels of both KYNA and ANA in stroke patients. There was also a positive correlation between ANA and KYNA at days 1, 2, 3 and 7. It was however expected that there would be a negative correlation between 3HANA and ANA, as the patient group had a lower level of 3HANA and a higher level of ANA, with the ratio 3HANA: ANA also showing significant differences between patients and controls, but 3HANA and ANA only correlated at day 1. There was a negative correlation between the K:T ratio and the 3HANA: ANA ratio at days 2 and 3, showing that activation of the pathway does influence this ratio. For at least 4 of the 6 time points, neopterin correlated negatively with tryptophan, positively with kynurenine and the K:T ratio, and positively with KYNA. Neopterin also correlated with ANA at 2 time points. ESR also correlated with the K:T ratio at day 1, giving more evidence that inflammation and kynurenine pathway activation are related in stroke patients. A correlation between neopterin and lipid peroxidation may be expected, but no correlation was observed. Lipid peroxidation correlated negatively with the 3HANA: ANA ratio at days 2, 3 and 7, but only correlated negatively with 3HANA itself at 1 time point and correlated positively with ANA at 2 time points. A lower 3HANA: ANA ratio may suggest increased oxidative stress in the stroke patient, indicated by the higher level of lipid peroxidation products.

For the control subjects, the K:T ratio correlated with KYNA and neopterin at all 3 time points and with ANA at 2 time points (Table 6-5). KYNA and ANA also correlated. This demonstrates that in healthy subjects, even small increases in inflammatory status occur in conjunction with increased metabolism via the kynurenine pathway.

There were correlations between age and neopterin for 3 of the 6 days for stroke patients (Table 6-4) and for all 3 days for the controls (Table 6-5). There were also correlations between age and K:T ratio for 3 out the 6 time points for patients and for 2 of the 3 time points for controls. KYNA also correlated with age but at fewer time points: significant correlations at day 2 for patients and at days 7 and 14 for controls.

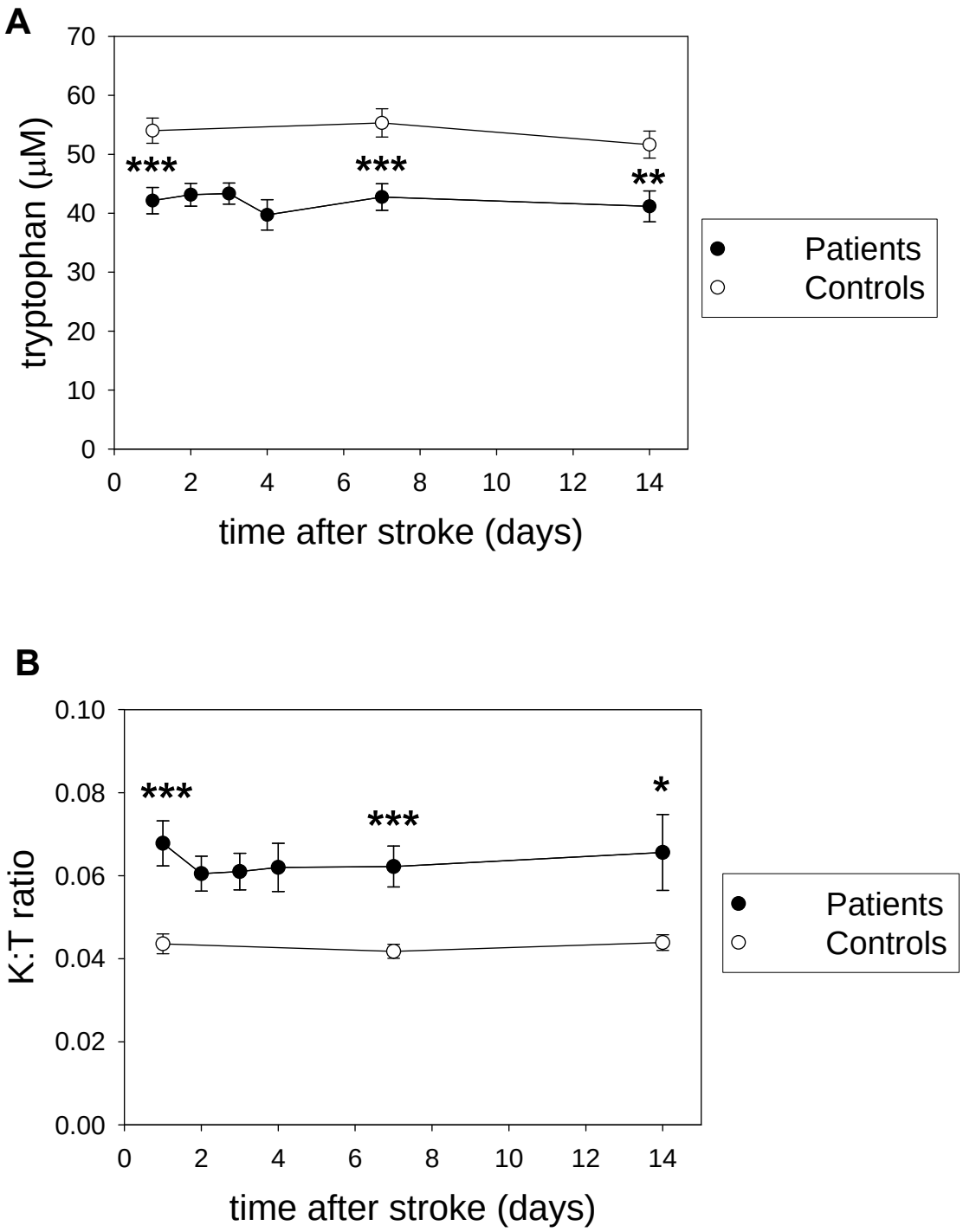


Figure 6-1 Changes in the levels of tryptophan (A) and in the kynurenine: tryptophan (K:T) ratio (B) over the 14 days following the stroke for patients or over a period of 14 days for controls. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

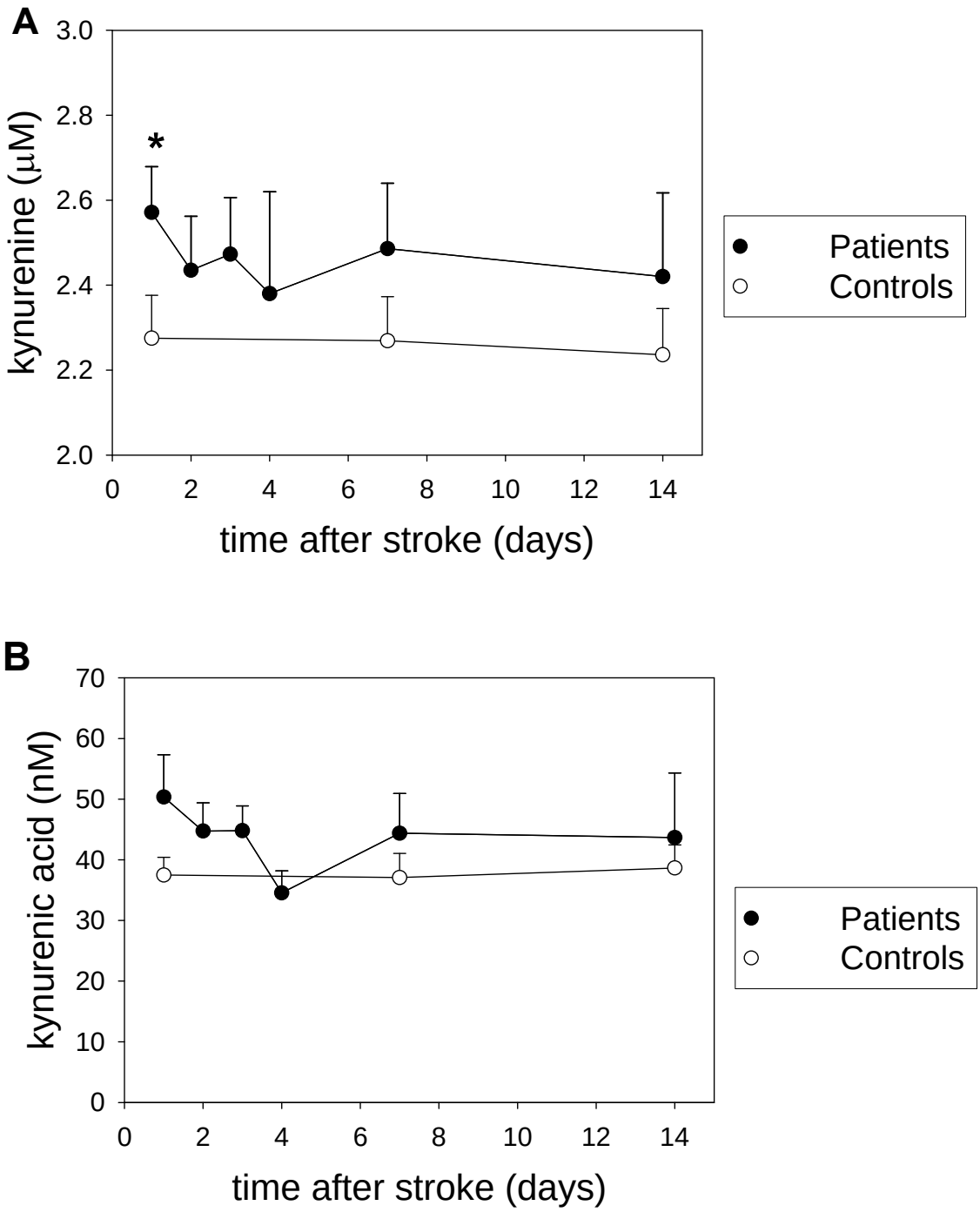


Figure 6-2 Changes in the levels of kynurenine (A) and kynurenic acid (B) over the 14 days following the stroke for patients or over a period of 14 days for controls. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$.

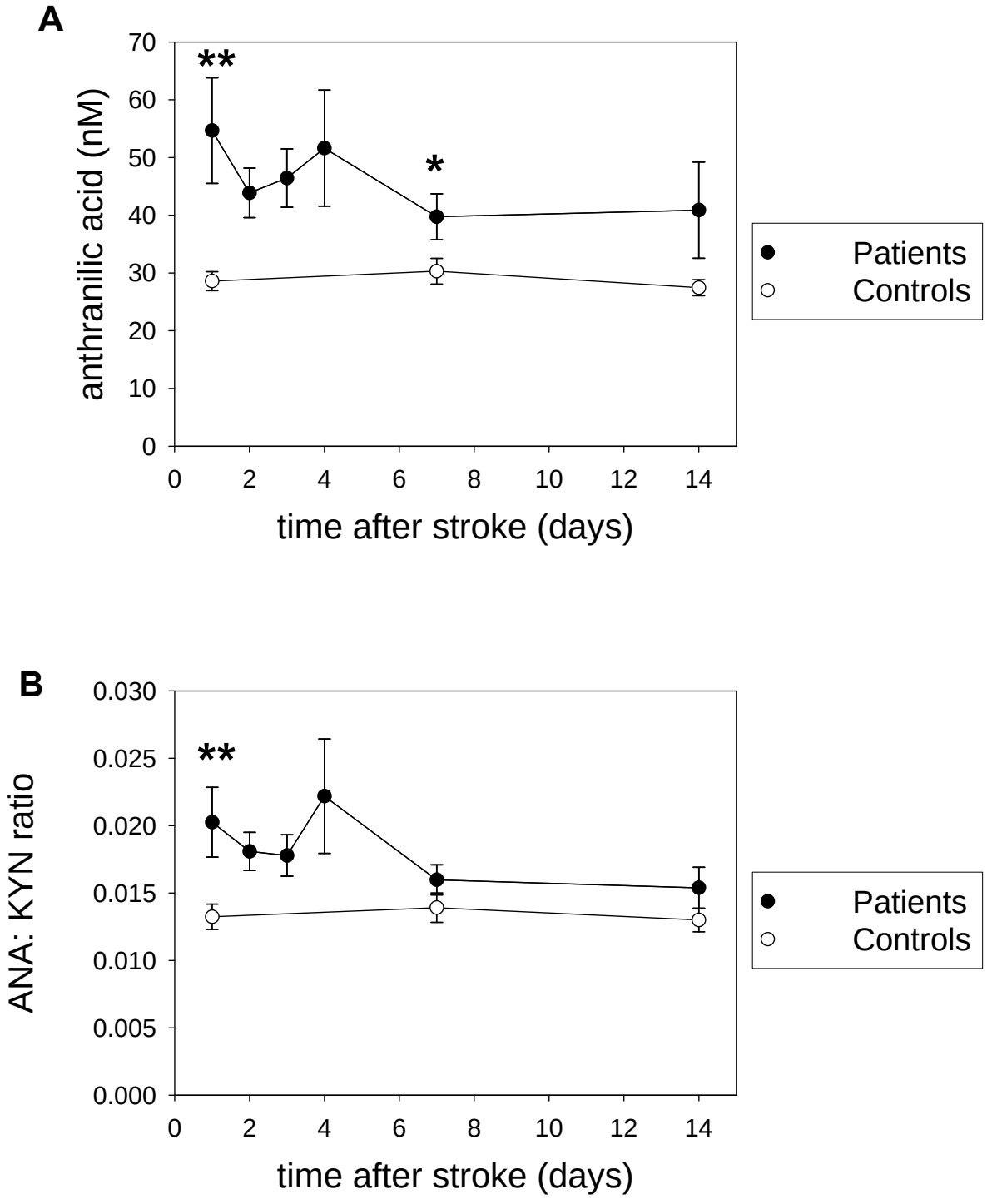


Figure 6-3 Changes in the levels of anthranilic acid (A) and in the anthranilic acid : kynurenine (ANA: KYN) ratio (B) over the 14 days following the stroke for patients or over a period of 14 days for controls. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$.

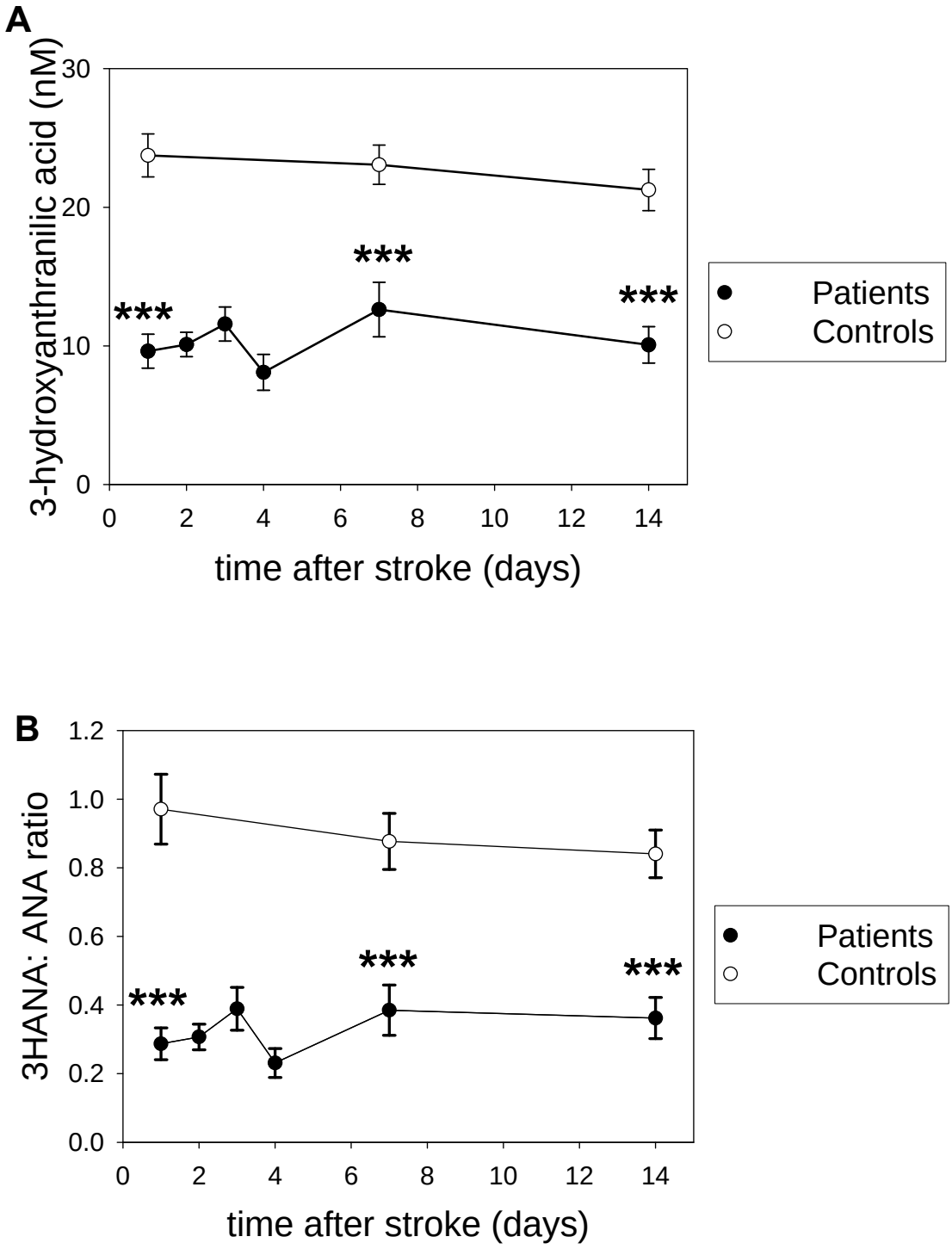


Figure 6-4 Changes in the levels of 3-hydroxyanthranilic acid (A), and in the 3-hydroxyanthranilic acid: anthranilic acid (3HANA: ANA) ratio (B) over the 14 days following the stroke for patients and over a period of 14 days for controls. Values are mean ± SEM. Significant differences between patients and controls: *** $p<0.001$.

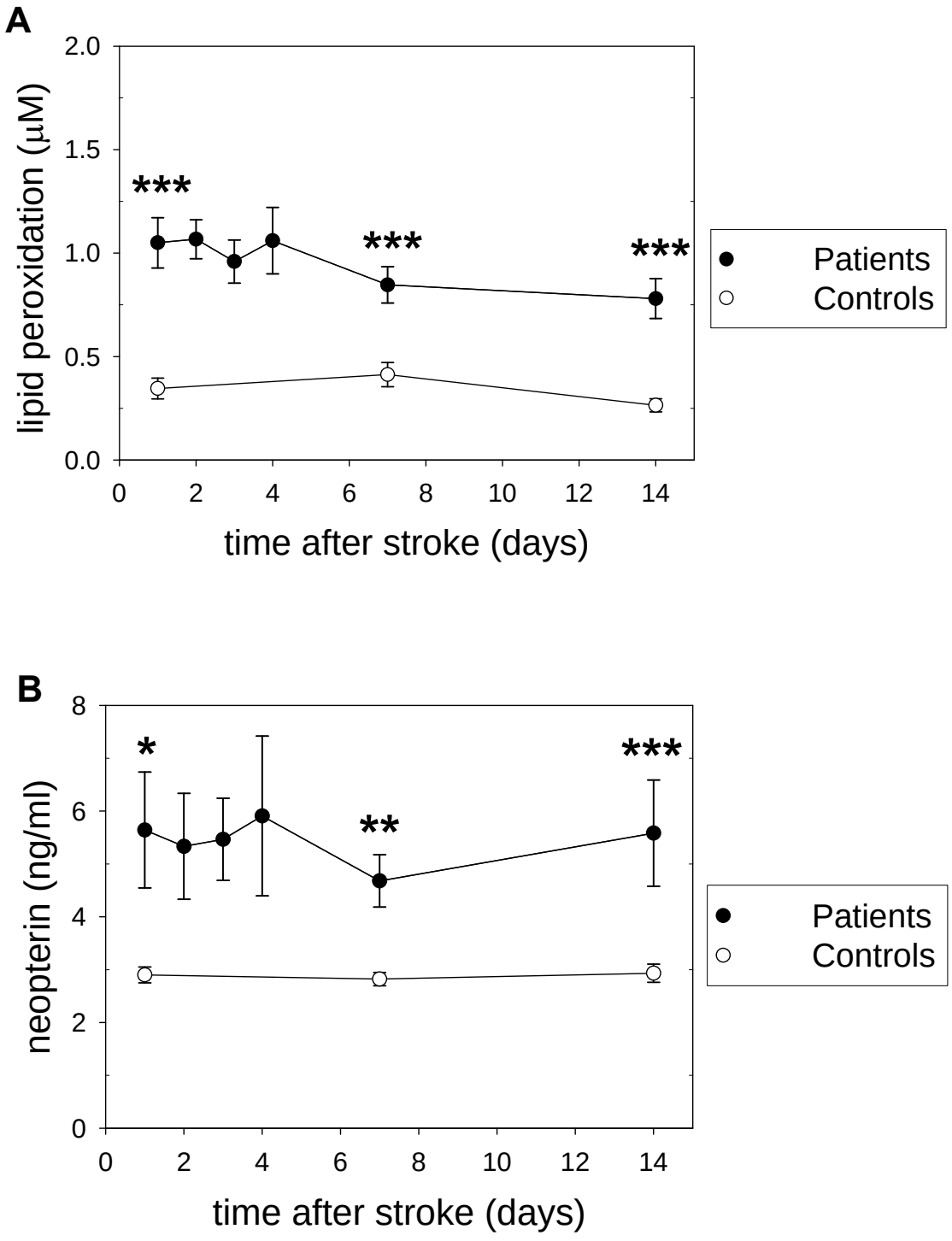


Figure 6-5 Changes in the levels of lipid peroxidation products (A) and neopterin (B) over the 14 days following the stroke for patients and over a period of 14 days for controls. Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 6-3 Spearman correlations between the estimated volume of brain damage from the CT scans and levels of the kynurenines, neopterin and lipid peroxidation for stroke patients. Significant correlations: *p<0.05, **p<0.01. No significant correlation: ns. Where there were no significant correlations at any of the time points, the results were not tabulated. (TRYPT - tryptophan, NEOPT - neopterin, LP - lipid peroxidation).

Positive (+) and Negative (-) Correlations with estimated volume of brain damage from CT scans:		**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**	**Day 14**
TRYPT	+	ns	ns	*	ns	ns	ns
3HANA	-	*	**	ns	ns	*	ns
3HANA: ANA	-	*	**	*	ns	*	ns
NEOPT	-	*	ns	ns	ns	ns	ns
LP	+	ns	ns	*	*	ns	ns

Table 6-4 Spearman correlations between the kynurenines and neopterin, lipid peroxidation and age for stroke patients. Significant correlations: *p<0.05, **p<0.01, ***p<0.001. No significant correlation: ns. Where there were no significant correlations at any of the time points, the results were not tabulated, unless a correlation was expected. (KYN - kynurenine)

Positive (+) and Negative (-) Correlations between:			**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**	**Day 14**
KYN	**KYNA**	+	***	***	**	ns	***	*
KYN	**ANA**	+	***	**	***	ns	***	**
K:T	**KYNA**	+	***	***	**	ns	***	ns
K:T	**ANA**	+	*	ns	***	ns	**	ns
KYNA	**ANA**	+	***	*	**	ns	**	ns
3HANA	**ANA**	-	*	ns	ns	ns	ns	ns
K:T	**3HANA: ANA**	-	ns	*	***	ns	ns	ns
NEOPT	**TRYPT**	-	**	*	**	ns	ns	*
NEOPT	**KYN**	+	*	*	*	ns	***	*
NEOPT	**K:T**	+	***	***	***	*	***	***
ESR	**K:T**	+	**					
NEOPT	**KYNA**	+	*	*	*	ns	*	*
NEOPT	**ANA**	+	ns	ns	*	ns	*	ns
NEOPT	**LP**	ns	ns	ns	ns	ns	ns	ns
LP	**3HANA: ANA**	-	ns	*	***	ns	*	ns
LP	**3HANA**	-	ns	*	ns	ns	ns	ns
LP	**ANA**	+	ns	ns	***	ns	*	ns
AGE	**K:T**	+	*	*	ns	*	ns	ns
AGE	**KYNA**	+	ns	*	ns	ns	ns	ns
AGE	**NEOPT**	+	ns	**	***	ns	*	ns

Table 6-5 Spearman correlations between the kynurenines and neopterin, lipid peroxidation and age for controls. Significant correlations: *p<0.05, **p<0.01, ***p<0.001. No significant correlation: ns. Where there were no significant correlations at any of the time points, the results were not tabulated, unless a correlation was of particular interest.

Positive (+) and Negative (-) Correlations between:			**Day 1**	**Day 7**	**Day 14**
KYN	**KYNA**	+	***	**	**
KYN	**ANA**	+	ns	ns	*
K:T	**KYNA**	+	*	**	***
K:T	**ANA**	+	**	ns	**
KYNA	**ANA**	+	ns	**	**
3HANA	**ANA**	ns	ns	ns	ns
K:T	**3HANA: ANA**	-	*	ns	ns
NEOPT	**TRYPT**	+	ns	*	*
NEOPT	**KYN**	+	***	***	***
NEOPT	**K:T**	+	**	*	**
NEOPT	**KYNA**	ns	ns	ns	ns
NEOPT	**ANA**	+	ns	ns	*
NEOPT	**LP**	ns	ns	ns	ns
LP	**3HANA: ANA**	-	*	ns	ns
LP	**3HANA**	-	ns	ns	**
LP	**ANA**	ns	ns	ns	ns
AGE	**K:T**	+	**	*	*
AGE	**KA**	+	ns	*	*
AGE	**NEOPT**	+	***	*	***

6.3.2 Comparison of Ischaemic and Haemorrhagic Patients

The results were split into the subgroups, ischaemic (n = 36) and haemorrhagic stroke (n = 9), and the two groups compared. It was unfortunate that the 2 groups contained such different numbers of patients, but a statistical comparison was still regarded as useful to determine any large differences between the 2 groups. For tryptophan, there was a trend for ischaemic patients to have higher tryptophan levels than the haemorrhagic patients but this was only significant at day 7 (Figure 6-6A). Ischaemic and haemorrhagic patients had lower tryptophan levels than the controls, but now that the data has been split into smaller groups, this was only significant at day 7 for ischaemic patients and at days 7 and 14 for the haemorrhagic group. When K:T ratios were examined, there were no significant differences between the ischaemic and haemorrhagic groups, and ratios remained significantly higher than the controls for both subgroups at each time point (day 1, 7 and 14) (Figure 6-6B).

There were no significant differences between the ischaemic and haemorrhagic groups for kynurenine, KYNA, 3HANA and ANA. However when the 3HANA: ANA ratio was examined, the haemorrhagic patients had a significantly lower ratio than the ischaemic patients at days 1 and 2 after the stroke (Figure 6-6C). The 3HANA: ANA ratios for both subgroups remained significantly lower than the controls throughout the study. There were also no significant differences between the ischaemic and haemorrhagic groups for lipid peroxidation and neopterin.

ANOVA showed that there were no significant effects with time after stroke for either the ischaemic or haemorrhagic groups, for all the analytes.

Both the ischaemic and haemorrhagic groups showed the same trends as the combined patients group compared with controls for all the measurements.

For the ischaemic patients only, correlations between the lesion volumes from the CT scans and the kynurenines, neopterin, lipid peroxidation and ESR were examined. The correlations which were significant were very similar to those that were significant for the combined patient group. There were significant correlations with lesion volume for 3HANA at days 2 and 7, for 3HANA: ANA ratio at days 1, 2 and 7 and for lipid peroxidation at day 3.

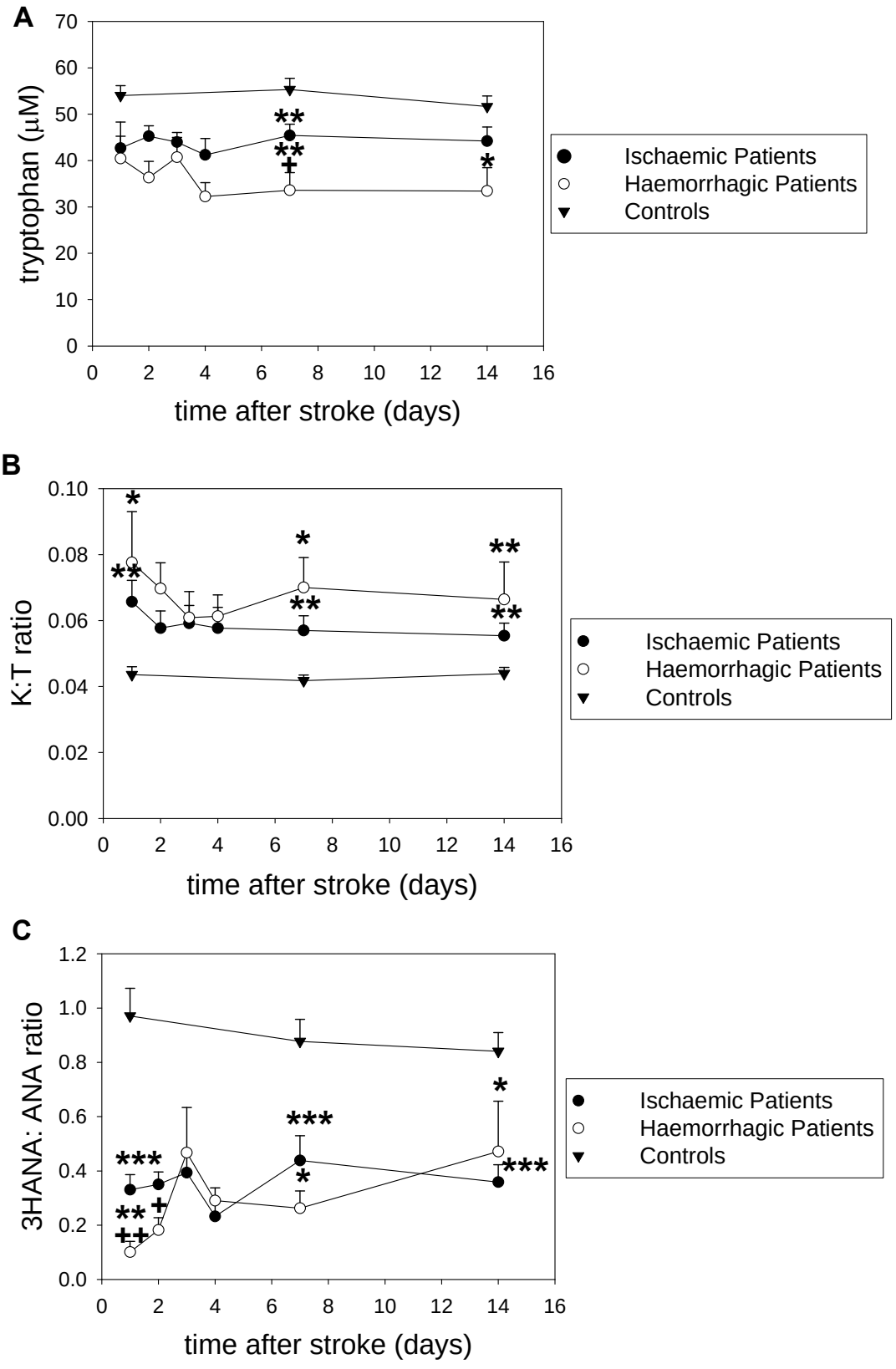


Figure 6-6 Comparison of ischaemic and haemorrhagic patients for tryptophan levels (A), kynurenine: tryptophan (K:T) ratios (B) and 3-hydroxyanthranilic acid: anthranilic acid (3HANA: ANA) ratios (C). Values are mean ± SEM. Significant differences between patients and controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significant differences between ischaemic and haemorrhagic groups: + $p<0.05$, ++ $p<0.01$.

6.3.3 Severity of the Stroke

Patients were divided into three subgroups: those who died within 3 weeks of the stroke (n=12), those who were discharged from hospital during the study (n=12) and those who remained in hospital for the duration of the study (n=26). The levels of tryptophan were not significantly different between the 3 groups. At day 1, patients who were able to go home during the study had lower kynurenine levels than patients who died or remained in hospital (Figure 6-7A). There was a trend that patients who died after the stroke had higher kynurenine levels at the other time points, although these differences were not significant. Kynurenic acid levels were significantly higher for the patients who died (Figure 6-7B). At days 1 and 3, these KYNA levels were significantly higher compared with those who went home during the study, and at days 3 and 7, significantly higher than for the patients who remained in hospital. At day 1, patients who died also had higher KYNA levels than the controls. Anthranilic acid levels also tended to be higher for patients who died (Figure 6-8A). Similar to the kynurenine results, this was only significant at day 1, where patients who died had higher ANA levels than those who were discharged. Also at day 1, the patients who died and those who remained in hospital had higher ANA levels than controls, while the patients who went home were not significantly different from controls. Patients who died also tended to have lower 3HANA levels at days 1, 2, 3 and 4, but this was only significant at day 4, with both the patients who died and those who remained in hospital having lower 3HANA levels than those who went home. All subgroups remained significantly lower in 3HANA concentrations than the controls at both day 1 and day 7. Neopterin and lipid peroxidation levels were not significantly different between these subgroups.

The subgroups of patients who died, those who were discharged and those who remained in hospital were further divided depending on whether the stroke was ischaemic and haemorrhagic, resulting in very small sample numbers. There were no effects that could be specifically attributed to the ischaemic or haemorrhagic group.

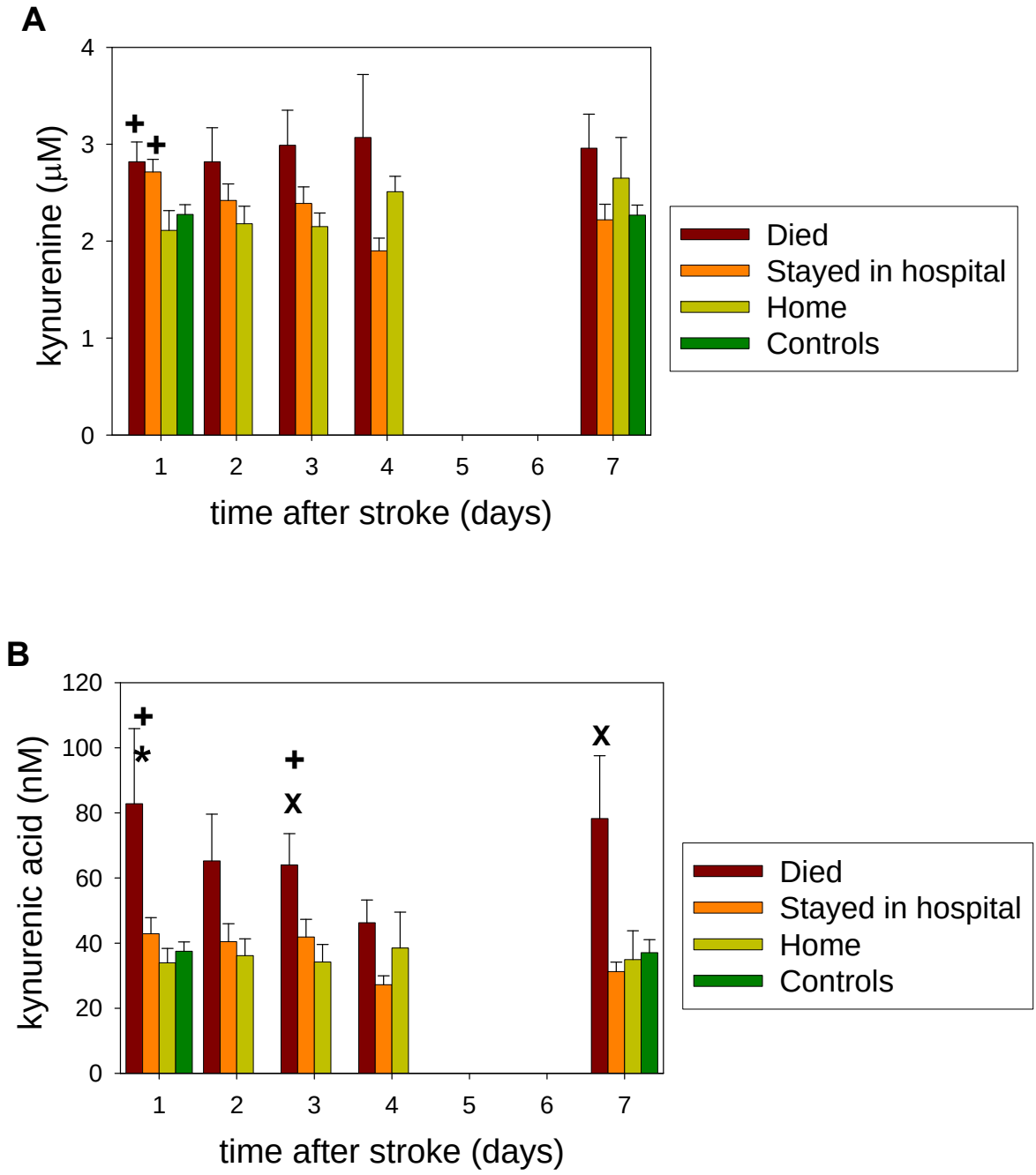


Figure 6-7 Effect of severity of the stroke on the levels of kynurenine (A) and kynurenic acid (B). Values are mean ± SEM. Significant differences compared with controls: * $p<0.05$. Significant differences compared with patients who were able to go home during the study: + $p< 0.05$. Significant differences compared with patients who stayed in hospital during the study: X $p<0.05$.

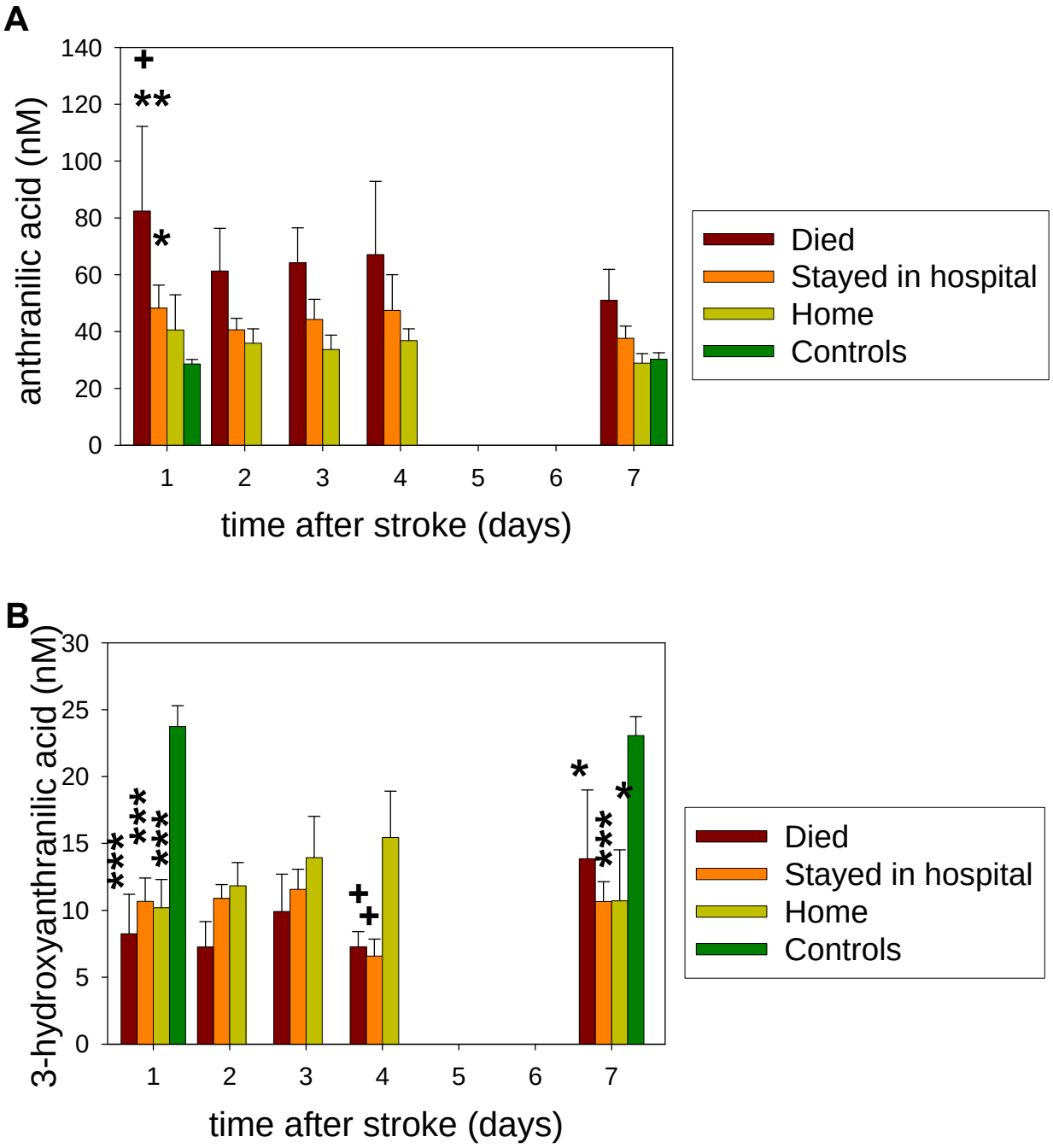


Figure 6-8 Effect of the severity of the stroke on the levels of anthranilic acid (A) and 3-hydroxyanthranilic acid (B). Values are mean ± SEM. Significant differences compared with controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significant differences compared with patients who were able to go home during the study: + $p< 0.05$.

6.3.4 Effect of Ischaemic Patients taking Aspirin on the Levels of Kynurenines, Neopterin and Lipid Peroxidation Products

Ischaemic patients who were taking aspirin (n = 14) were compared with those who were not (n = 11), using unpaired two-tailed t tests, for samples taken 1, 2, 3 and 7 days after the stroke. For tryptophan, kynurenine, KYNA, 3HANA, ANA and lipid peroxidation, there were no significant effects of taking aspirin at each time point. It appears that there were lower levels of neopterin for patients taking aspirin compared with those not taking aspirin, at 1, 2 and 3 days after the stroke, significant at day 3 (Figure 6-9). Neopterin is a marker of inflammation, so this result may be expected as aspirin has anti-inflammatory properties. However, a much higher dose of aspirin than was administered to these patients is expected to be required before it has anti-inflammatory properties. There are extremely large error bars for neopterin levels in patients not taking aspirin at days 1, 2 and 3 (Figure 6-9). On closer examination of the data, there are 3 patients with extremely high neopterin levels of between 12 and 34 ng/ml, which are skewing the data and suggesting this effect of aspirin on neopterin levels. Details of these 3 patients were studied, and it was noted that none of them died during the study, but all 3 were on antibiotics and therefore may have had a severe infection. However many other patients were also taking antibiotics. The effect that patients not taking aspirin have higher neopterin levels is skewed by the results from 3 patients and therefore is unlikely to be valid.

6.3.5 Effect of Other Drugs on the Levels of Kynurenines, Neopterin and Lipid Peroxidation Products

5 patients were taking glucocorticoids and 6 were taking antidepressants. For both subgroups, t tests were carried out to examine if there were any differences in tryptophan, kynurenine, KYNA, 3HANA, ANA, neopterin and lipid peroxidation between patients taking these drugs and the other patients. Neither of these drugs caused significant differences in any of the measurements.

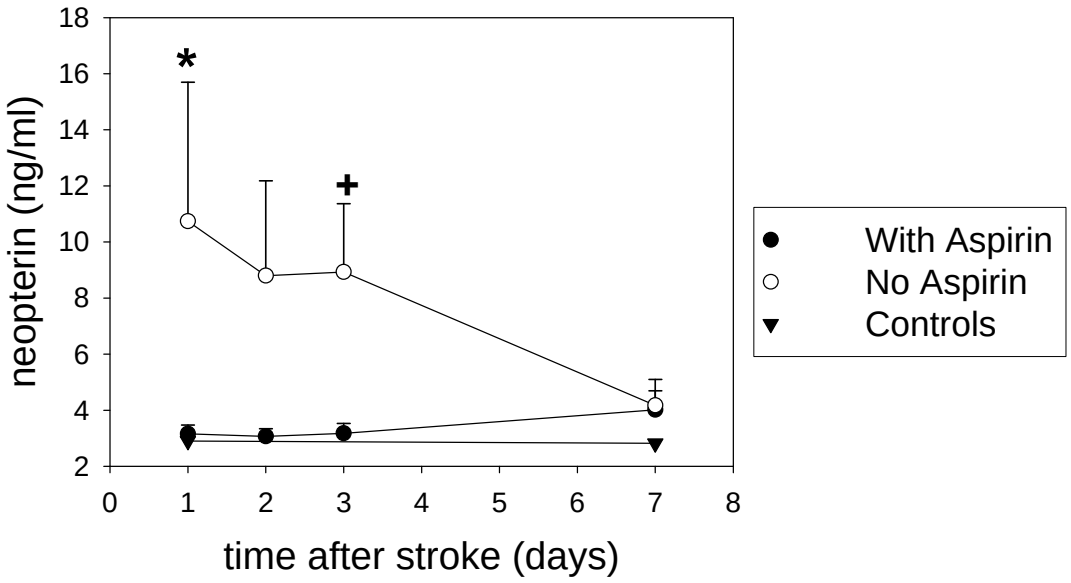


Figure 6-9 Comparison between the neopterin levels for ischaemic patients who were taking aspirin throughout the study and those who were not taking aspirin. Values are mean ± SEM. Significant differences compared with controls: * $p<0.05$. Significant differences between patients who were taking aspirin and those who were not: + $p< 0.05$.

6.4 Discussion

6.4.1 Tryptophan, Kynurenine and IDO Activity

The results of the stroke study, in particular the higher K:T ratios for patients compared with controls, suggest that the kynurenine pathway was activated within 24 hours of the stroke occurring. Within the first 24 hours, neopterin and lipid peroxidation products were also elevated compared with controls. The elevated neopterin indicates that the inflammatory response was increased within 24 hours of the stroke and therefore pro-inflammatory cytokines could have activated IDO, the initial enzyme of the kynurenine pathway. Neopterin levels correlated with the K:T ratio at all 6 time points after the stroke, suggesting that a stroke patient with greater inflammation showed increased activation of IDO. This is in agreement with other human diseases where increased neopterin levels correlated with increased IDO activity, both neopterin and IDO being stimulated by the pro-inflammatory cytokine, IFN-γ (Schroecksnadel *et al*., 2005).

Activation of the kynurenine pathway, via induced IDO activity, was apparent throughout the 14 days of the study, as the K:T ratio remained significantly elevated in patients, demonstrating that more kynurenine was being produced from the available tryptophan. However, it is possible that tryptophan levels in the blood are lower in the patients due to dietary intake, as patients after a stroke are likely to be eating less than the healthy controls. In an ideal study, diet would have been monitored. However, lower tryptophan levels in patients would be expected to produce less kynurenine and increased or similar levels of kynurenine were determined. Additional evidence for activation of the pathway was the significantly higher levels of ANA measured in the patient samples.

6.4.2 Inflammation in Stroke

The high levels of neopterin support the view that inflammation occurs rapidly after a stroke (Barone and Feuerstein, 1999; Skinner *et al*., 2006). The continued elevation of neopterin demonstrates that the inflammatory cascade occurs for many hours or days following a stroke, which may be important as there is much evidence that inflammation contributes to delayed brain injury (Dirnagl *et al*., 1999). Activated monocytes and macrophages produce and release neopterin and therefore neopterin levels reflect the degree of macrophage activation. Neopterin itself can trigger pro-inflammatory events and has been related to the production of nitric oxide resulting in oxidative stress (Hoffmann

and Schobersberger, 2004). However, there were no correlations between neopterin and lipid peroxidation in this study.

6.4.3 Timescale for Kynurenine Pathway Activation

MRI scans for stroke patients have shown that substantial enlargement of the ischaemic lesion can occur later than 24 hours after onset, although enlargement often occurs earlier (Baird *et al*., 1997). In human PET studies, potentially viable penumbra was present up to 48 hours after stroke (Heiss *et al*., 1992). In rats, after an MCAO, neurones in the penumbra showed accelerated ischaemic damage between 8 and 12 hours after occlusion, with complete loss of neurones after 10 days (Dereski *et al*., 1993). The levels of all the kynurenines in our study, as well as neopterin and lipid peroxidation, remained similar throughout the 14 days after the stroke.

The activated kynurenine pathway within 24 hours of the stroke is not as expected from the gerbil model of ischaemia, where no increases in IDO activity or QUIN levels were observed in the brain until at least 2 days following the ischaemic insult, with maximum levels after 4 days (Saito *et al*., 1993b). This was suggested to be due to the infiltrating macrophages having high levels of IDO, and maximum macrophage levels were present in the gerbil brain 4 days after ischaemia. Inflammation results in neutrophils, followed by macrophages and monocytes migrating from the blood into the ischaemic brain, the latter two cell types becoming the predominant cells five to seven days after ischaemia in animal models (Dirnagl *et al*., 1999). Resident brain cells are also involved in the inflammatory response: microglia are activated and astrocytes become enlarged, four to six hours after ischaemia. Twenty-four hours after an MCAO, there is much microglial activation in the ischaemic brain, particularly in the penumbra (Dirnagl *et al*., 1999). Activated microglia also express elevated levels of kynurenine pathway enzymes, including IDO, and, as well as macrophages, are able to produce QUIN from tryptophan in the brain (Heyes *et al*., 1996). As macrophage activation in the blood has been shown to occur earlier than in the brain, demonstrated by the elevated neopterin levels at 24 hours after the stroke, IDO activity would also be expected to increase in the blood at the same time. The discrepancy between the timescale of IDO activation in this study and in the gerbil ischaemic model may also be due to the species difference between humans and gerbils.

The results of this study could suggest that macrophage activation following a stroke results in increased IDO activity. However, as IDO activity appears to be activated at the first timepoint in the study, it cannot be ruled out that the kynurenine pathway may be

activated before the stroke and contribute to the stroke occurring. Humans are more susceptible to stroke if they have high blood pressure, atherosclerosis, heart disease, high cholesterol or diabetes and these underlying pathologies may include an activated kynurenine pathway. This contrasts with animal models of stroke where healthy animals are given an ischaemic insult, and the brain damage then starts to develop.

6.4.4 Kynurenic Acid

There was no significant increase in KYNA levels following the strokes, showing similarity with the gerbil ischaemic stroke model, where neither KYNA nor KAT were elevated (Saito *et al*., 1993b).

6.4.5 3-Hydroxyanthranilic Acid, Lipid Peroxidation and Oxidative Stress

The increased levels of lipid peroxidation in stroke patients are consistent with substantial evidence that oxidative stress is involved in the cerebral tissue damage occurring after ischaemia (Love, 1999; Chan, 2001). Oxidative stress was also shown to contribute to DNA damage and brain injury in a study of experimental intracerebral haemorrhage in rats (Nakamura *et al*., 2005). Macrophages and microglia, activated by inflammation, can produce neurotoxins including nitric oxide and reactive oxygen species (Dirnagl *et al*., 1999), which may be contributing to the increased lipid peroxidation.

Of particular interest in this study was the decreased levels of 3HANA in the stroke patients. 3HANA is redox-active and can generate free radicals, contributing to oxidative stress. In human monocyte-derived cells in culture, apoptosis has been shown to be induced by IDO activity stimulated with IFN-γ (Morita *et al*., 2001). It was shown that 3HANA, but not 3HKYN or QUIN, induced apoptosis in these cells, and apoptosis was enhanced by the presence of redox-active metals (Fe^{2+} or Mn^{2+} ions). It was suggested that different cell types are susceptible to toxicity by different kynurenine pathway metabolites. 3HANA can undergo autoxidation which ultimately produces reactive oxygen species (Dykens *et al*, 1987), oxidative stress and apoptosis (Okuda *et al*., 1998). Such metabolism of 3HANA may cause the decreased concentration of this metabolite in the blood and may contribute to the increased lipid peroxidation in the stroke patients.

The redox activity of 3HANA depends on the presence of other redox-active compounds, and in some situations, 3HANA can also act as a scavenger of peroxyl radicals, as in the

initial step of autoxidation, 3HANA is oxidised (Christen *et al*., 1990; Leipnitz *et al*., 2007). This suggests an alternative hypothesis in which the lower levels of 3HANA offer less antioxidant activity, resulting in less protection against oxidative stress for the stroke patients. The lower levels of 3HANA may also suggest that increased levels of QUIN have been formed, depleting its substrate, 3HANA. However, there is evidence for abundant 3HAO in normal brain tissue (Saito *et al*., 1993b) and therefore this should not limit the production of QUIN from 3HANA in the controls. It may be more likely that higher levels of 3HANA would produce more QUIN. This cannot be clarified here as the levels of QUIN were not determined in this study.

The importance of the reduced levels of 3HANA in stroke patients was emphasised by the correlations between 3HANA and the volume of brain damage observed in the CT scan at 3 of the 6 time points in the study.

6.4.6 Anthranilic Acid

Activation of the kynurenine pathway may be expected to increase the concentration of ANA, as ANA can be produced from kynurenine by the enzyme, kynureninase. However, the preferred route for kynurenine metabolism under physiological conditions has been established as being via 3HKYN, as the enzyme involved in this reaction, KMO, has a high affinity for the substrate, kynurenine (Bender and McCreanor, 1982). 3HKYN can then be converted to 3HANA. ANA may produce 3HANA but no enzyme for this metabolism has been identified so the only possible mechanism is by direct hydroxylation. This has only been demonstrated in a single study where a high level of ANA added to rat cortical brain slices was shown to produce 3HANA (Baran and Schwarcz, 1990). In the stroke patients, the high levels of ANA and the low levels of 3HANA may suggest that this metabolism is blocked, although most of the 3HANA would be expected to be produced from 3HKYN. Unfortunately 3HKYN was not measured in this study, as levels were expected to be close to the LoD of the method. Knowledge of the concentrations of 3HKYN in stroke patients would have helped to clarify the possible metabolic routes involved. There was only a correlation between 3HANA and ANA in patients at day 1, making it unclear whether these metabolites behave independently or not.

ANA has been shown to demonstrate some neuroprotective properties by partially blocking QUIN toxicity when co-injected with QUIN into the rat brain (Jhamandas *et al*., 1990), but to a much lesser extent than the NMDA receptor antagonist, KYNA. The

increased levels of ANA in the stroke patients may be a compensatory response to the neurotoxic damage to the cells of the penumbra in the stroke patients.

ANA can also interact with copper to form an anti-inflammatory complex. This complex acts as a hydroxyl radical-inactivating ligand, able to remove the highly injurious hydroxyl radicals at inflammatory sites (Miche *et al*., 1997; Gaubert *et al*., 2000). The high levels of ANA generated following stroke may be an anti-inflammatory response, offering some protection from oxidative stress.

6.4.7 3-Hydroxyanthranilic Acid : Anthranilic Acid

Interestingly, in other work by our group for patients with osteoporosis, decreases in the levels of 3HANA and increases in the levels of ANA in blood were also observed (Forrest *et al*., 2006). These changes were reversed after 2 years of drug treatment to levels similar to controls, which suggests the potential for drug treatment to reverse these changes in the stroke patients.

The correlations with lipid peroxidation are stronger for the 3HANA: ANA ratio (significant at 3 out of the 6 time points) than for the 3HANA levels themselves (only significant at 1 time point), suggesting that the ratio is key. Similarly, the 3HANA: ANA ratio changes appear to result from the activation of the kynurenine pathway as the ratios correlated with the ratios of K:T at days 2 and 3 in patients. The importance of the ratio of 3HANA: ANA in this stroke study is demonstrated by the significant correlations with the volume of brain damage from the CT scans, at 4 of the 6 time points, being significant at one more time than 3HANA itself.

Interestingly, 3HANA: ANA, 3HANA itself and lipid peroxidation showed the most significant correlations with the volume of brain damage from the CT scans, suggesting that these are important parameters involved in the brain damage caused by stroke. The correlations of brain damage with lipid peroxidation were not at any of the same time points as the correlations with 3HANA and only at one of the same time points with 3HANA: ANA ratio. The relationship between lipid peroxidation and 3HANA or the 3HANA: ANA ratio was not entirely clear from these correlations.

One possible mechanism for the increased levels of ANA and decreased levels of 3HANA is that there may be a lack of the enzyme, KMO, which produces 3HKYN followed by 3HANA from kynurenine. This could also account for the higher ANA levels measured,

as kynurenine could not be metabolised via its preferred route to 3HKYN and could therefore be metabolised via ANA by the enzyme, kynureninase. Such increased metabolism was significant at day 1 after the stroke when the ratio of ANA: KYN was higher in patients compared with controls. KMO availability has been demonstrated to be a potential variable in kynurenine pathway metabolism. When a systemic immune stimulus was given to gerbils, there was a lack of KMO in the brain and hence QUIN could not be produced from tryptophan or kynurenine within the brain (Saito *et al*., 1993c). However, KMO inhibition has been shown to increase KYNA concentrations (Cozzi *et al*., 1999), and KYNA levels were similar in stroke and control patients. A reduction in KMO activity suggests that 3HKYN and QUIN would also be lower after stroke, and therefore does not suggest a role for any of these three neurotoxic products of the pathway. However, several KMO inhibitors have shown neuroprotection in animal models of ischaemia (Cozzi *et al*., 1999), suggesting that KMO activity is high in stroke. Therefore, it is unlikely that reduced KMO activity was the cause of the high levels of ANA and low levels of 3HANA in the blood.

The 3HANA: ANA ratio may not be important, with the altered concentrations of 3HANA and ANA in stroke patients occurring independently of each other. A more plausible explanation for the increased ANA in stroke patients may be an increased kynureninase activity.

6.4.8 Ischaemic and Haemorrhagic Stroke

Patients with symptoms of stroke and who were diagnosed with stroke were included in the study to achieve the maximum number of samples at all 6 time points examined. The CT scan clarified whether the stroke was ischaemic or haemorrhagic. The differences in severity of the stroke for the ischaemic patients gave further variability in the patient group. In an ideal study, a large number of patients with ischaemic stroke of similar severity would be used. However, such a group of patients were not available for this work. As this study is a preliminary study for examining if the kynurenine pathway is altered in human stroke, further studies would have to be more carefully controlled.

Although an ischaemic stroke involves a blockage of a major brain artery and a haemorrhagic stroke involves a blood vessel bursting in the brain, many of the same mechanisms of brain damage occur for both: excitotoxicity, oxidative stress, inflammation and cell death. Both have an area surrounding the core site of damage which may be able to regain function: the penumbra in ischaemic stroke (Dirnagl *et al*., 1999) and the

perihaematoma in haemorrhagic stroke (Greenberg, 2007). There is also a serious risk of oedema in both types of stroke.

The results from this study show that there are very few differences between the ischaemic and haemorrhagic groups, which gives some justification for using the combined patient group. Both groups showed the same differences compared with controls. The trend for lower tryptophan in the haemorrhagic group may be because these patients were generally more severely affected by the stroke and therefore there was more activation of the kynurenine pathway. The severity may also account for the lower 3HANA: ANA ratios at days 1 and 2 in the haemorrhagic group. Four of the nine (44%) haemorrhagic patients died within 3 weeks of the stroke, with none of the other five being sent home during the study. For the larger ischaemic group, patients were less severely affected, with only 14% (5 patients) dying within 3 weeks of the stroke and 12 patients able to go home during the study, with the remaining 19 staying in hospital.

6.4.9 Severity of Stroke

The severity of the stroke resulted in changes in the levels of the kynurenines. In particular, patients who died within 3 weeks of the stroke had high levels of KYNA. Although the haemorrhagic patients tended to be more severely affected by the stroke, the ischaemic group, when analysed separately, still showed significant increases in KYNA levels for patients who died. As well as the neuroprotective properties of KYNA, this metabolite also has been shown to have detrimental physiological characteristics. High KYNA levels have been shown to lower the efficacy of mitochondrial ATP synthesis (Baran *et al*., 2003). This effect was demonstrated in rat heart tissue, but the concentrations of KYNA affecting mitochondria function were 125μM, much higher than the increased levels of KYNA in the stroke patients, which were up to 100nM. However, these concentrations of 100nM are in blood and there is the possibility that local intracellular levels in the brain may be much higher. Disturbances in mitochondrial function have been shown to be involved in stroke (Dirnagl *et al*., 1999).

Patients more severely affected by the stroke, dying within 3 weeks, appeared to have a more activated kynurenine pathway compared to those who were discharged within 2 weeks of the stroke. There were trends suggesting that kynurenine and ANA levels were elevated in patients who died, compared with those who went home. The 3HANA concentrations also tended to be lower for the patients who died compared to those who were discharged. These differences in kynurenine, ANA and 3HANA concentrations were

only significant at one of the 5 time points examined. The lack of significance may be due to the small sample numbers resulting from subgrouping the patients. The differences observed between the total patient group and controls were exaggerated in the patients who died. However, the concentrations of neopterin and lipid peroxidation did not change. The changes in other kynurenines suggest that the increase in KYNA in patients who died was due to abnormal kynurenine pathway metabolism. However, it is feasible that KYNA was produced by a different route, possibly from indole-3-pyruvic acid (IPA). In the presence of free radicals, the enol tautomer of IPA can form KYNA (Bartolini *et al*., 2003). There was no evidence for the presence of increased free radicals for patients who died, as there was no change in lipid peroxidation.

6.4.10 Aspirin and Other Drugs

Aspirin (acetylsalicylic acid), at low doses (approximately 75mg daily), is often given to stroke patients to try to enhance blood perfusion after a stroke, acting by inhibiting the aggregation of blood platelets. Sherman (2004) examined the results from two very large clinical trials where the role of aspirin administration after acute ischaemic stroke was evaluated. It was concluded that aspirin has a significant benefit in reducing stroke recurrence when given within 48 hours.

Aspirin however can also be used as a non-steroidal anti-inflammatory drug, but a much higher dose is required for the anti-inflammatory effect (usually 1-4g daily (British Medical Association and Royal Pharmaceutical Society of Great Britain, 1999)). Anti-inflammatory drugs, such as aspirin, can interfere with the kynurenine pathway, as they can modulate cytokine production and reduce IDO stimulation by cytokines (Schroecksnadel *et al*., 2005). Sodium salicylate has been shown to inhibit TDO activity in the liver and hence reduce kynurenine pathway activity (Badawy, 1982). However, high levels of aspirin (1.8 – 2.7g) cause a significant increase in the concentration of free tryptophan by displacing the amino acid from serum albumin and thus increasing kynurenine pathway activity resulting in lower plasma tryptophan levels (Smith and Lakatos, 1971; Cotlier, 1981). It was therefore unclear if aspirin would affect the kynurenine pathway in our study.

It was concluded that the potential artefact of the study, that some patients were taking aspirin and others were not, was not significantly affecting the measurements made. Both patients taking aspirin and those not taking aspirin showed similar differences between patients and controls as was observed with the complete patient set, for all the analytes.

Glucocorticoids are also used to suppress inflammation, and therefore may alter cytokine production, IDO activation and kynurenine pathway metabolism. In this study, there was no evidence that glucocorticoids affected the kynurenines measured.

Antidepressants could potentially interfere with metabolism via the kynurenine pathway as many antidepressants inhibit TDO activity and kynurenine pathway metabolism, resulting in elevated brain tryptophan concentrations and increased 5HT synthesis in rats (Badawy and Evans, 1982). Some antidepressants are selective serotonin re-uptake inhibitors (SSRI) and hence maintain elevated levels of 5HT. This may have an impact in the production of 5HT from tryptophan and could therefore affect the concentrations of tryptophan and its availability for kynurenine pathway metabolism. In our study, there was no evidence for patients taking antidepressants having different levels of kynurenines in their blood.

6.4.11 Conclusions

It has been shown that the kynurenine pathway is activated following a stroke, in conjunction with increased inflammation and oxidative stress. A major finding from this study is the much decreased concentrations of 3HANA in the blood of the stroke patients. This is possibly related to the increased levels of ANA. The concentrations of 3HANA and the 3HANA: ANA ratio correlated negatively with the stroke lesion volumes, demonstrating that these kynurenine changes in the blood are related to the brain damage caused by the stroke. 3HANA and the 3HANA: ANA ratio also correlated negatively with lipid peroxidation, suggesting that 3HANA may be depleted due to autoxidation which can contribute to oxidative stress. Alternatively, as 3HANA is redox-active and can also act as an antioxidant, less 3HANA may be produced from ANA in stroke patients, resulting in less free radical scavenging ability and less protection from oxidative stress. Patients who died within 3 weeks of having a stroke showed higher concentrations of KYNA in their blood, which suggests that KYNA may either act as an NMDA receptor antagonist attempting to compensate for the excitotoxic damage in the brain, or act in reducing mitochondrial ATP synthesis, hence contributing to neuronal cell death.

7 General Discussion

7.1 Alterations of the Kynurenine Pathway in the Blood of Patients with Huntington's Disease, Chronic Brain Injury and Acute Stroke

In HD, there is much evidence that the activation of the kynurenine pathway is involved in the mechanisms of neurodegeneration, investigated primarily in human post-mortem brain tissue (Beal *et al*., 1990; Pearson and Reynolds, 1992; Guidetti *et al*., 2004). There is much less published literature about the role of kynurenines in TBI or stroke, although after TBI, human CSF had high levels of QUIN (Sinz *et al*., 1998), and the kynurenine pathway was altered in animal models of ischaemic stroke (Saito *et al*., 1993b; Cozzi *et al*., 1999).

The results from all three studies showed alterations in kynurenine pathway metabolism, measured in the blood. The inflammatory marker, neopterin, was increased in all three studies, with levels being higher in the acute stroke patients than for the patients with chronic disorders (HD and chronic brain injury). As may be expected, this demonstrates a greater inflammatory response immediately after acute injury. As neopterin levels were increased, changes in the kynurenine pathway were assumed to be related to IDO activity and not TDO activity, as IDO was activated in response to an inflammatory stimulus and TDO activity was expected to be more constant (Heyes *et al*., 1993). In each study, there was evidence for increased IDO activity, by significant increases in the K:T ratio at baseline for the HD and chronic brain injury patients, and after stroke. Interestingly, the difference between the K:T ratios for patients and controls in each study was approximately 0.02, demonstrating a similar degree of activation of IDO immediately after an acute stroke compared with late stage neurodegeneration (HD) or chronic brain injury. However, the mechanism for the increased IDO activity differs, as the results from HD and chronic brain injury patients demonstrated an increase in kynurenine concentrations in the blood, with no change in tryptophan levels, whereas the stroke patients showed a decrease in tryptophan levels, with an increase in kynurenine levels only at day 1. This suggests a difference between acute and chronic injury. A possible explanation is that in acute injury, tryptophan is degraded by IDO and quickly metabolised down the pathway, compared with in chronic injury, where there has been sustained IDO activity which may produce a build up of kynurenine, with tryptophan levels having had time to renormalise.

Although there was increased IDO activity, there were no similar increases in KYNA levels in the blood in any of the three studies. For stroke patients, the general trend was for increased KYNA concentrations in the blood, although this was not significant, and there was no change in KAT activity. The chronic brain injury patients, however, had KYNA levels which were lower than controls at the initial baseline. For the chronic disorders (HD and chronic brain injury), there was low KAT activity in the patients, as the higher levels of kynurenine did not produce higher levels of KYNA. This is in agreement with Beal *et al*. (1990), where a significant increase in the ratio KYN: KYNA was demonstrated in the post-mortem brains of HD patients. In addition, the activity of the KAT II enzyme was decreased, with its Km increased 3-fold, in the putamen of HD post-mortem brains, although the decreases in KYNA levels were not significantly different from control brains (Jauch *et al*., 1995). In our HD and chronic brain injury studies, the decreased KAT activity was very clearly accompanied by increased IDO activity. The inflammatory response, which increased IDO activity, may be the cause of KAT inactivation, and this was supported by the negative correlations between the KYNA: KYN ratio and neopterin at baseline in HD patients and the KYNA: KYN ratio and CRP in the chronic brain injury patients. The inactivity of KAT in these chronic disorders suggests a slow progressive deficiency in KYNA over time. As KYNA can block NMDA receptor activation, KYNA deficiency could induce excitotoxicity, potentially leading to slowly developing neurodegeneration, as hypothesised by Beal *et al*. (1990).

One of the most interesting and unexpected findings of this work was the significantly reduced levels of 3HANA in the blood of patients with HD, chronic brain injury and acute stroke, compared with their respective controls. Lower levels of 3HKYN were also found in the blood of the HD and chronic brain injury patients, but 3HKYN was not measured in the stroke study. It has been established in several studies that 3HKYN is increased in post-mortem striatum and cerebral cortex in late stage HD (Pearson and Reynolds, 1992; Guidetti *et al*., 2000). Therefore, it was unexpected to find decreased levels of 3HKYN and 3HANA in the blood of the HD patients. To maintain high levels of 3HKYN in the brain, blood pools of 3HKYN may have been depleted. Indeed, 3HKYN can cross the blood-brain barrier via the large neutral amino acid transporter (Fukui *et al.*, 1991) and 3HKYN has selective uptake into certain brain regions, particularly the striatum (Okuda *et al*., 1998), where much of the neurodegeneration in HD occurs. In the brain, 3HANA is produced from 3HKYN, as 3HANA does not easily cross the blood-brain barrier (Fukui *et al.*, 1991). The reduced levels of 3HANA in the blood may be due to reduced production from the low levels of 3HKYN. If indeed high levels of 3HKYN and 3HANA were present in the brain, they could have led to increased autoxidation of these metabolites

which resulted in enhanced production of reactive oxygen species. As the pathway is dynamic, it may be expected that more 3HKYN and 3HANA would form after their degradation by autoxidation, or otherwise, more could be taken up from the blood.

Alternatively, the low levels of 3HKYN and 3HANA in the blood may have been the consequence of degradation via autoxidation in the periphery. 3HKYN and 3HANA may have autoxidised at a faster rate than they could be produced. As autoxidation of 3HKYN or 3HANA produces the superoxide anion (Dykens *et al*., 1987; Goldstein *et a*l., 2000), such autoxidation may promote further IDO activity as IDO uses the superoxide anion as a co-substrate. The removal of the superoxide product of 3HANA autoxidation has been shown to accelerate the reaction (Dykens *et al*., 1987), and hence if IDO removes the superoxide anion, the autoxidation process will be promoted. This would result in continuing activation of the kynurenine pathway and continuing production of reactive oxygen species in these three disorders. 3HKYN and 3HANA toxicity results in neuronal cell death by apoptosis (Chiarugi *et al*., 2001; Morita *et al*., 2001). As apoptosis has been implicated as a major process of neuronal cell death in HD (Portera-Cailliau *et al*., 1995; Ona *et al*., 1999), in stroke (Hara *et al*., 1997; Moroni *et al*., 2001) and in TBI (Yakovlev *et al*., 1997), oxidative stress induced by 3HKYN and 3HANA is a possible mechanism for neuronal damage in HD, chronic brain injury and stroke. The large 3-4 fold increases in lipid peroxidation levels in all three disorders studied, suggested that oxidative stress was an important mechanism of neuronal damage.

The major effect of 3HKYN and 3HANA *in vivo* is toxicity caused by oxidative stress. Intracerebroventricular administration of 3HKYN has been shown to be toxic in mice, and causes seizures (Lapin, 1981). Nakagami *et al*. (1996) demonstrated that intrastriatal injection of 3HKYN induced tissue damage in rats. The antioxidant, N-acetyl-L-cysteine, reduced the 3HKYN damage, but the NMDA receptor inhibitor, MK-801, failed to protect the brain from 3HKYN toxicity. This suggests that oxidative stress, and not the metabolism of 3HKYN into QUIN, is causing the toxicity. Jhamandas *et al.* (1990) showed that a single injection of 3HANA into the nucleus basalis magnocellularis in rats, produced a large decrease in the survival of cholinergic neurones. Transition metal ions are required for 3HKYN and 3HANA toxicity (Eastman and Guilarte, 1990; Okuda *et al*., 1996), and even at trace levels of these ions, 3HKYN and 3HANA have been shown to generate superoxide (Goldstein *et al*., 2000). *In vivo*, the low levels of transition metal ions that are required for 3HKYN and 3HANA toxicity should be available. Inflammation has been shown in all three disorders studied, and as it induces acidosis which liberates copper and iron from proteins, may increase the availability of iron and copper to enhance

the 3HKYN and 3HANA toxicity (Goldstein *et al*., 2000). As the antioxidant properties of 3HKYN and 3HANA have been demonstrated only *in vitro*, it is more probable that the 3HKYN and 3HANA effects in our studies were related to their pro-oxidant activity.

For the stroke study, ANA was also measured and showed increased concentrations in the blood of the stroke patients. ANA can react with copper and the resulting anthranilic acid-Cu^{2+} complex has anti-inflammatory properties, which can remove the highly injurious hydroxyl radicals at inflammatory sites (Miche *et al*., 1997; Gaubert *et al*., 2000). ANA has also been shown to partially protect against QUIN–induced neuronal damage (Jhamandas *et al*., 1990), which may indeed be due to the ability of ANA to remove hydroxyl radicals, as these are produced during QUIN-induced toxicity (Goda *et al*., 1996). The stroke patients showed positive correlations between ANA and lipid peroxidation, suggesting that higher levels of lipid peroxidation induced ANA synthesis. The increase in ANA levels in the stroke patients may be a compensatory response to the neurotoxicity, which could offer protection from oxidative stress.

The importance of the low levels of 3HANA in the stroke patients has been demonstrated by the negative correlations between 3HANA concentrations and the lesion volumes from the CT scans. Lipid peroxidation correlated positively with lesion volumes in the stroke patients, suggesting that oxidative stress was a major factor in neuronal cell death in stroke.

The high concentrations of ANA together with the low levels of 3HANA in the blood of the stroke patients suggested that the metabolism of ANA to produce 3HANA was blocked. However, most of the 3HANA produced was expected to be metabolised from 3HKYN (Bender and McCreanor, 1982). The ratios of 3HANA: ANA, which may indicate the extent of direct hydroxylation of ANA, showed negative correlations with lipid peroxidation and lesion volume at more time points than for 3HANA alone. In addition, the K:T ratio correlated negatively with the 3HANA: ANA ratio but not with 3HANA or ANA themselves, indicating that the extent of kynurenine pathway activation was related to decreased 3HANA: ANA ratios. One possible mechanism for the increased levels of ANA and decreased levels of 3HANA is reduced activity of the enzyme, KMO, which produces 3HKYN, followed by 3HANA, from kynurenine. This could also account for the higher ANA levels measured, as kynurenine could not be metabolised via its preferred route to 3HKYN and would therefore be metabolised to ANA by the enzyme, kynureninase. This hypothesis suggests that the levels of 3HANA and ANA would be closely related but these metabolites only correlated at 1 of the 6 time points in the stroke study, indicating that they may behave more independently of each other. It was

unfortunate that 3HKYN and QUIN were not measured for the stroke patients, as the HD and brain injury results suggest that 3HKYN concentrations in the blood may also have been reduced.

It is possible that the reduced levels of 3HKYN and 3HANA contribute to increased activity of the immune system, as both metabolites can regulate the immune system by suppressing T cell proliferation (Bauer *et al*., 2005). Enhancing the immune response would include increased activation of microglia, which are thought to be instrumental in producing brain damage in HD (Sapp *et al*., 2001), stroke (Dirnagl *et al*., 1999) and TBI (Sahuquillo *et al*., 2001). In addition, more macrophages would be able to infiltrate the brain and contribute to the neuronal damage in stroke and TBI (Dirnagl *et al*., 1999; Sahuquillo *et al*., 2001). Activated microglia and macrophages produce neurotoxins including cytokines, nitric oxide and reactive oxygen species.

Guidetti *et al*. (2004) demonstrated that QUIN was increased in post-mortem brains from patients with grade 1 HD but not in brains of later stages of HD. This suggests that the neurotoxicity is initiated by the excitotoxin, QUIN. Our results agreed with this work as there was no change in QUIN in the blood of patients at late stage HD. However it was unexpected that the reduced levels of 3HKYN and 3HANA in the blood of HD and chronic brain injury patients did not reduce the levels of QUIN produced. This supports the hypothesis that 3HKYN and 3HANA are produced in the blood following activation of the kynurenine pathway, but are rapidly transported (3HKYN only) to the brain, autoxidised or metabolised to QUIN, resulting in low levels being detected.

There is some evidence from our studies to support the hypothesis of Guidetti *et al*. (2000) for HD, which states that increased metabolism through the 3HKYN/QUIN branch compared with the KYNA branch is involved in HD neurodegeneration. At the initial baseline for both HD and chronic brain injury, the QUIN: KYNA ratios were slightly higher in patients compared with controls, although they were not significant. These higher QUIN: KYNA ratios, together with the highly significant reduction in KAT activity for both studies, offer some support for this HD hypothesis.

7.2 Kinetics of Tryptophan Metabolism in Huntington's Disease and Chronic Brain Injury Patients

As the kynurenine pathway is the major metabolic pathway for tryptophan in most tissues, after protein synthesis, oral tryptophan depletion and loading resulted in large changes in all the kynurenine metabolites measured in the blood.

With the small number of samples in the HD and chronic brain injury studies, there will be fewer significant effects than would be expected with a larger sample group. However, it was remarkable that the HD patients and the chronic brain injury patients showed extremely similar tryptophan metabolism and kinetics of the kynurenine pathway on depletion and loading. Indeed, even the neopterin and lipid peroxidation responses were very similar at baseline and after loading. These studies have demonstrated strong similarities in kynurenine pathway metabolism between the neurodegenerative HD and chronic brain injury, mainly initiated by a traumatic injury.

Both HD and chronic brain injury patients may use less tryptophan for other metabolic processes, such as protein synthesis, than controls, as both groups of patients had higher levels of tryptophan at 5 hours after trytophan depletion. Further evidence of this was observed in the chronic brain injury patients, where the tryptophan load also produced higher levels of tryptophan than the controls. Although it was only at these extreme conditions of depletion or loading that this difference in tryptophan metabolism was observed, the increased tryptophan metabolism to kynurenine would be expected to have the opposite effect and lower the tryptophan levels in these patients' samples. Therefore, it is hypothesised that HD and chronic brain injury patients use less tryptophan for other metabolic processes, such as protein synthesis.

The HD patients demonstrated increased IDO activity (measured as K:T ratios) throughout the depleting and loading protocols, although the significance was lost after loading, but tryptophan depletion caused the K:T ratios for the chronic brain injury patients to become much more similar to controls at the second baseline and lower than controls after loading. This suggests that there is a stronger link with kynurenine pathway activity in HD than in chronic brain injury. Although neopterin levels were higher in HD and chronic brain injury patients than in controls at baseline, there were no significant differences between neopterin levels after loading, suggesting that this similar level of inflammation after loading will produce similar IDO activity in both patients and controls. Werner *et al*.

(1988) demonstrated that increasing tryptophan concentrations from 50 to 500μM inhibited IDO activity in human cells in culture. Tryptophan loading may inhibit IDO in the chronic brain injury patients to a greater extent than in the HD patients. A tryptophan load will enhance TDO activity (Schutz *et al*., 1972), but this would be expected to be similar for both patients and controls.

The hypothesis that inflammation may be inactivating KAT in the patients with these chronic disorders was suggested by the increased neopterin levels in the patients at baseline. The KAT activity in the chronic brain injury patients, which had been inactivated at baseline, was increased compared with controls after loading. On loading, this increase in KAT activity paralleled decreases in IDO activity, confirming the strong relationship between the activities of these enzymes. For HD, the low KAT activity at baseline and after depletion was returned to levels similar to the controls after loading. After loading, where there was no inactivation of KAT, neopterin levels were similar in patients and controls, supporting the hypothesis that inflammation may be inactivating KAT.

It was interesting that the elevated levels of 3HKYN and 3HANA after the tryptophan loading, remained lower than controls for both the HD and chronic brain injury patients, although there were marked changes in IDO and KAT activity.

For HD and chronic brain injury patients after an oral tryptophan load, there was a delayed response for increases in QUIN compared with all the other kynurenine metabolites, including KYNA. In agreement with this effect, Jauch *et al*. (1993) demonstrated that serum QUIN levels rose much more slowly than KYNA levels with intravenous administration of kynurenine in rhesus monkeys. KYNA levels peaked within 10 minutes of the kynurenine load, and then gradually declined over the remainder of the 4 hours of the study, whereas the QUIN concentrations increased steadily to reach a maximum after approximately 90 minutes, with levels being maintained at this maximum until the end of the study at 4 hours. The decline in KYNA levels in the blood was suggested to be due to efficient urinary excretion. The rapid decline in KYNA levels observed in the blood for these monkeys may suggest that for our study, the maximum levels of all the tryptophan metabolites in the blood may not occur at 5 or 7 hours after oral loading. However, there were significant increases at 5 hours after the tryptophan load, with concentrations having decreased between 5 and 7 hours for all the kynurenines measured, with the exception of QUIN. This suggests similar response times to the tryptophan load for the measured kynurenines except QUIN.

Often the differences in the levels of tryptophan metabolites between patients and controls were similar at baseline and after tryptophan depletion. Depletion of tryptophan therefore did not appear to have a beneficial effect compared to a normal diet. Tryptophan loading increases the levels of the neurotoxic tryptophan metabolites in both patients and controls, but it is the balance between the neurotoxic kynurenines (QUIN, 3HKYN and 3HANA) and the neuroprotective KYNA, that may be more important than the actual concentrations. After tryptophan loading, the potentially detrimental effects of increased IDO activity and reduced KAT activity were lost for both the HD and chronic brain injury patients. This suggests that tryptophan loading may be beneficial for HD and chronic brain injury patients. However, the QUIN data indicated a possible build up of QUIN over time after loading in HD patients and therefore tryptophan loading cannot be justified as a neuroprotective measure.

7.3 Tryptophan Loading Induces Oxidative Stress

An unexpected result from the HD and chronic brain injury studies was an increase in lipid peroxidation after tryptophan loading in the healthy control subjects. Tryptophan administration has previously been reported to increase lipid peroxidation in rats (Aviram *et al*., 1991). In conjunction with this, an increase in the inflammatory response was also shown after loading in the healthy controls. These results suggest that tryptophan loading may be harmful and therefore care should be taken if tryptophan loading is being used to investigate the effects of increasing 5HT in the brain in relation to depression, or in a diet such as the Atkins diet, which involves a chronically raised intake of tryptophan. It should be noted that the high dose of 6g tryptophan resulted in increased lipid peroxidation in this study, whereas much lower doses (1g tryptophan taken three times a day) are more common for use in depression studies (Shaw *et al*., 2002). A tryptophan load can be used in the treatment of depression as patients seek the use of a 'natural alternative' to prescribed antidepressants, but has the side effects of nausea and gastrointestinal distress (Shaw *et al*., 2002). Tryptophan ingestion has been associated with Eosinophilia –Myalgia Syndrome, which affected nearly 1500 people in 1989 and led to over 30 deaths. However, this outbreak was traced to a contaminant in the biotechnologically manufactured tryptophan from one manufacturing source (Simat *et al*., 1999) and there remains no evidence for toxicity associated with tryptophan ingestion.

7.4 Changes in Kynurenines with Time and Severity of Stroke

The initial alterations in the kynurenine pathway observed one day after the stroke did not change over the next 14 days. In addition, the elevated neopterin levels and lipid peroxidation remained very similar, although there was a trend for the lipid peroxidation to decrease slightly over time. From animal studies, a delay in the maximum kynurenine pathway activity was expected, which peaked at 4 days and then returned to normal within 14 days (Saito *et al*., 1993b) but this was not seen in our study. As macrophages infiltrating the brain have been implicated as a major contributor to the increased IDO activity and increased levels of QUIN in the brain following ischaemia in animals (Saito *et al*., 1993c), macrophage activation may take place earlier in the blood and therefore changes in kynurenine pathway activity in the brains of the patients may still be delayed. The immediate effect of the alterations in the kynurenine pathway for stroke patients is consistent with the failure of NMDA antagonists in stroke clinical trials. One reason for the failure was attributed to these drugs not being able to be administered to the patients until many hours after the stroke, as time had elapsed between the stroke occurrence and the patients reaching hospital (Green and Shuaib, 2006). Alternatively, as IDO activity was demonstrated in the initial blood sample one day after stroke, patients may have greater IDO activity before the stroke occurred, which may contribute to the patients' susceptibility to stroke.

Various excitotoxic compounds, including the NMDA receptor agonist QUIN, have been shown to induce KYNA synthesis in response to excitotoxicity (Wu *et al*., 1992; Guidetti *et al*., 1997; Ceresoli-Borroni *et al*., 1999). Increased excitotoxicity may be a possible explanation for the higher levels of KYNA in the blood of patients who died within 3 weeks of having a stroke. As excitotoxicity generates free radicals, one possible mechanism for these increases in KYNA formation is from the generation of nitric oxide, as nitric oxide donors have been shown to increase the production of KYNA in cortical brain slices (Luchowski and Urbanska, 2007). Indeed, neopterin, which was elevated in this study, has been implicated in the production of nitric oxide, although neopterin levels were not increased in the patients who died. In addition, in the presence of oxygen free radicals, KYNA can be produced from IPA (Bartolini *et al*., 2003). Elevated levels of KYNA can cause excessive NMDA receptor blockade which may be related to the cardiovascular side effects observed with NMDA receptor inhibitors in stroke clinical trials (Dirnagl *et al*., 1999). Extremely high levels of KYNA (greater than 125μM) can reduce

mitochondrial ATP synthesis (Baran *et al*., 2003) and although much lower levels were observed in the patients who died in our study (mean at day 1 was 83nM), it is possible that local levels in the brain in these patients may be high enough to cause this effect.

7.5 Relationship Between Blood and Brain Levels of Kynurenines

Clearly, there are drawbacks to studying kynurenine pathway metabolites in the blood when it is their action in the CNS that is of primary concern. It is likely that the concentrations of kynurenines in the blood are partly due to tryptophan metabolism in the liver. However, if changes in kynurenine pathway activity can be observed in the blood in neurological disorders, blood samples would provide much more readily obtainable samples for further studies of these disorders and may provide useful markers for the disorders. It is important to examine living patients as opposed to post-mortem brains or animal models of a disease because potential therapies that could be developed from the results of this study would be applicable to living patients. Ideally, CSF would have been used in these studies but we could not justify ethically taking CSF from these patients.

Tryptophan, kynurenine and 3HKYN can be transported between the plasma and the brain by the large neutral amino acid carrier (Fukui *et al*., 1991). With healthy primates (rhesus monkeys), intravenous administration of kynurenine was shown to increase levels of KYNA and QUIN in both the serum and the CSF (Jauch *et al*., 1993). This demonstrates that all the enzymes required to synthesis QUIN and KYNA from kynurenine were active in both the periphery and the brains of healthy primates. Since the synthesis of QUIN and KYNA were elevated in proportion to the dose of kynurenine (maximum 200mg/kg), none of the enzymes were saturated with substrate, suggesting that the availability of kynurenine is the main determinant for the production of QUIN and KYNA in both the periphery and the brain, in agreement with IDO/TDO being the rate-limiting step of the kynurenine pathway. A systemic tryptophan load can produce large increases in QUIN in the rat brain (Heyes and Markey, 1988; During *et al*., 1989). KYNA is known to be readily produced from kynurenine in the human brain (Okuno and Kido, 1991). As systemic administration of tryptophan and kynurenine has been shown to alter the kynurenine pathway in both the blood and brain, and tryptophan, kynurenine and 3HKYN can be transported between the blood and the brain, the levels of kynurenine metabolites in the blood were expected to reflect the levels in the brain.

A difference between the concentrations of kynurenines in the blood and brain was shown by the different rates of KYNA and QUIN production and excretion in the blood of rhesus monkeys (Jauch *et al*., 1993). Following intravenous kynurenine administration, the steady decline in KYNA concentrations in the blood was attributed to efficient urinary excretion of KYNA. There is also active organic acid efflux out of the CNS, which maintains the lower levels of kynurenines in the brain than in the blood, and has been demonstrated to reduce the concentrations of KYNA and QUIN in the brain (Miller *et al*., 1992; Morrison *et al*., 1999). With breakdown of the blood-brain barrier, which has been demonstrated in ischaemia and TBI (Skinner *et al*., 2006; Habgood *et al*., 2007), the small kynurenines would be able to pass more freely from the blood to the brain, particularly in the areas of brain damage. Organic acid efflux and breakdown of the blood-brain barrier may influence the proportional relationship between the kynurenine levels in the blood and brain.

Tryptophan in the blood can be free or bound to plasma albumin. It has a very high level of protein binding (about 95%) (Reilly *et al*., 1997). Most of the kynurenines may also bind to albumin in plasma. There have been contrasting reports for kynurenine binding, with Joseph and Kadam (1979) and Cangiano *et al*. (1999) showing that kynurenine did bind to albumin but Fukui *et al*. (1991) demonstrated no significant binding to plasma protein. The acids of the kynurenine pathway (KYNA, QUIN, ANA, 3HANA) have also been shown to bind to plasma albumin, however, at physiological pH and temperature, QUIN, but not the other acids, was in a free state (Fukui *et al*., 1991). Only unbound tryptophan and kynurenine pathway metabolites are available for transport across the blood-brain barrier (Fukui *et al*., 1991).

7.6 Comparison with Reported Concentrations of Kynurenines in Healthy Human Blood

The concentrations of kynurenines in healthy human blood measured in this project were compared with concentrations quoted in the literature. The mean tryptophan levels for the control plasma samples for the HD and chronic brain injury studies was 73.6 ± 3.5 μM (mean ± SEM), very similar to levels quoted in many papers in the literature (Heyes *et al*., 1994; Widner *et al*., 2000; Pertovaara *et al*., 2006). For the stroke study, the tryptophan levels were lower in the healthy controls (mean 54.0 ± 2.1 μM), but were still within the range quoted in the literature (Saito *et al*., 2000; Frick *et al*., 2004). The range of reported kynurenine concentrations in healthy human blood was from 1.35μM to 3.27μM (Joseph

and Risby, 1975; Heyes *et al*., 1994; Hervé *et al*., 1996), which was slightly higher than the mean level of 1.13 ± 0.28 μM for the control plasma samples for the HD and chronic brain injury patients, with the concentration of 2.28 ± 0.10 μM in the serum of the stroke controls being very similar to literature values. Levels of KYNA in healthy controls were 46.7 ± 0.6 nM and 37.5 ± 2.9 nM for the HD/chronic brain injury and stroke studies respectively, compared with reported serum KYNA levels of between 23nM and 59.6nM (Heyes *et al*., 1994; Hervé *et al*., 1996; Ilzecka *et al*., 2003; Fukushima *et al*., 2007). The reported concentration of 3HKYN in healthy human plasma was 32nM and another report quoted 383nM in human plasma (Pearson and Reynolds, 1991; Heyes *et al*., 1994). The method used in the HD and chronic brain injury studies showed a concentration of 232 ± 47 nM for healthy controls. The concentration of 3HANA in healthy controls for the HD/chronic brain injury studies was 177 ± 49 nM using the electrochemical method, but 23.7 ± 1.6 nM using the fluorescence method for the stroke controls. Literature levels of 3HANA in healthy human serum were 79nM and 24.2nM (Hervé *et al*., 1996; Arbatova *et al*., 2005). There are, therefore, some discrepancies between the levels of 3HKYN and 3HANA in human plasma or serum both in the literature and from the results of this project, which is likely to be due to the use of different methods of analysis. The electrochemical method used in this project for the determination of 3HKYN and 3HANA for the HD and chronic brain injury studies had low sensitivity and was superceded by the fluorescent method for measuring 3HANA concentrations for the stroke study. The levels of QUIN measured for the healthy controls in the HD and chronic brain injury studies was 1540 ± 830 nM, higher than reported concentrations of 432nM and 267nM (Heyes *et al*., 1991a; Vogelgesang *et al*., 1996). This high mean level of QUIN may have been due to the high variablility in the QUIN measurements for the HD and chronic brain injury studies.

7.7 Effect of Age

An additional observation from this study is that the age of the subjects correlated with the K:T ratio and KYNA concentrations, as well as with neopterin. Blood neopterin and K:T ratio have been shown to increase with the age of the subject (Frick *et al*., 2004). It was suggested that ageing in healthy people is associated with immune activation. In our work, age and neopterin correlated for the healthy controls in the stroke study and for the stroke and chronic brain injury patients. Similarly, there were correlations between age and the K:T ratio (a measure of IDO activity) for the stroke controls and patients at several time points. The stroke study incorporated a larger group of patients and controls than the HD

and chronic brain injury studies, which accounts for more correlations observed with age from this study. In addition, the patients and controls in the stroke study were older than in the HD and chronic brain injury studies, which suggests that old age may increase neopterin and K:T ratios at a faster rate than for younger people. In a study with nonagenarians, IDO activity, measured by the K:T ratio in the blood, was shown to be markedly increased and higher IDO levels were shown in the subjects who died in the following four years (Pertovaara *et al*., 2006). Increased IDO activity was suggested as a mechanism involved in the decline of T cell responses with old age.

Interestingly, there were correlations between age and KYNA levels, for HD and chronic brain injury controls and for stroke controls. This correlation was also observed for stroke, HD and brain injury patients but at fewer time points than for the controls. This agrees with Moroni *et al*. (1988) who reported that brain and blood levels of KYNA increase with age in rats. In human healthy controls for a stroke study where the subjects were elderly, serum KYNA levels correlated with age (Urbanska *et al*., 2006). As it was only the K:T ratio and KYNA levels that correlated with age at several time points, it suggests that on ageing, increased kynurenine is produced which is converted to KYNA rather than metabolising via the other branches of the kynurenine pathway. This is in direct contrast with the apparent inhibition of KAT by IDO shown in the HD and chronic brain injury studies. However, levels of QUIN in the CSF have also been reported to increase with age (Heyes *et al*., 1992a). The correlations with age demonstrate the importance of having age matched patients and controls in studies of the kynurenine pathway.

7.8 Future Work

One of the major findings of this work was the decreased concentrations of 3HKYN and 3HANA in the blood of patients with HD and chronic brain injury. There is much evidence demonstrating that 3HKYN is elevated in HD post-mortem human brain (Pearson and Reynolds, 1992; Guidetti *et al*., 2000), and an increase in 3HKYN was expected in the blood of HD patients. Most reported studies on kynurenines in HD have focused on post-mortem brain from human HD sufferers, although more recently the use of transgenic animal models of HD has begun (Guidetti *et al*., 2006). Little is known about blood levels of the kynurenines in HD. Future work is required to clarify if 3HKYN and 3HANA concentrations are elevated in the brain but decreased in the blood in HD. There are several genetic mouse models of HD, and using one of these would be useful to measure kynurenines in both blood and brain tissue. Guidetti *et al*. (2006) showed that 3HKYN

was increased in the striatum and cortex of three HD mouse models: transgenic R6/2 mice (expressing a short mutant htt fragment), YAC128 mice (expressing full length human htt) and Hdh^{Q92} and Hdh^{Q111} knock-in mice (expressing mutant htt with 92 or 111 glutamines respectively). The use of one of these mouse models would be ideal as 3HKYN has been shown to be increased in these brains, as it is in human HD brains. In addition to measuring 3HKYN and 3HANA, determining the levels of tryptophan, kynurenine and KYNA as before in both the blood and brains of a transgenic HD mouse model would be useful to examine changes occurring throughout the pathway. As there was a significant increase in ANA for the stroke patients, it would also be useful to measure ANA in the transgenic HD mouse model. Furthermore, other pathway metabolites, which have not been determined previously may also be important, in particular, 5-hydroxyanthranilic acid and picolinic acid. 5-Hydroxyanthranilic acid, as well as 3HANA, can be produced from ANA, and this may be especially relevant as the 3HANA levels decreased in HD patients' blood. Concentrations of picolinic acid, but not quinolinic acid, were significantly elevated in a mouse model of cerebral malaria (Clark *et al*., 2005), and it would be interesting to investigate if picolinic acid is important in HD. 5-Hydroxyanthranilic acid can be measured using HPLC and fluorescence detection and picolinic acid is determined by GC-MS.

As the ANA concentrations increased in the stroke study, bloods of the HD and chronic brain injury patients will be re-extracted and ANA levels will be determined, to investigate whether ANA was also increased for these patients.

It is ethically unjustifiable to take CSF from any of the patients involved in this work. Further studies using patients' blood samples would be useful to clarify the observations made in this project. The stroke study was a preliminary study and as abnormalities in the kynurenine pathway were observed, further work in patients with acute ischaemic stroke would be useful. Stroke patients are accessible and a large patient group, with ischaemic stroke of similar severity, could be assessed, with measurements taken at one specific time point within two weeks of the stroke. To determine if the lower levels of 3HKYN in blood from patients is due to less 3HKYN being produced by KMO from kynurenine, white blood cells, collected as the mononuclear cell layer, could be used to determine the expression of KMO in patients and healthy controls using Western blotting. The low levels of 3HKYN and 3HANA in patients may be due to autoxidation at a high rate, and to investigate this hypothesis, the products of autoxidation could be measured, involving HPLC with mass spectrometry for product identification. Oxidation products of 3HKYN that could be determined are xanthommatin, hydroxyxanthommatin and 4,6-

dihydroxyquinolinequinonecarboxylic acid (DHQCA) (Vazquez *et al*., 2000). Similarly, five oxidation products of 3HANA including cinnabarinic acid can be determined (Iwahashi, 1999). HPLC methods for these compounds could be developed in our laboratory.

This project has shown the potential importance of 3HKYN in neurological disorders, and therefore developing a more sensitive HPLC method for its determination would be very valuable. Further work will continue to pursue the SPE route for sample preparation for 3HKYN measurement in serum. 3HKYN levels could then be measured in the serum of the stroke patients.

QUIN levels appeared to be increasing at 7 hours after a tryptophan load was administered to HD and chronic brain injury patients, but no further blood samples were taken to determine if this increase continued. It would be interesting to assess if QUIN levels continued to rise in patients after a tryptophan load at later time points and to determine when QUIN levels started to decrease or return to normal. This would indicate how long the neurotoxin, QUIN, remains elevated in these patients after ingesting high concentrations of tryptophan.

7.9 Conclusions

It was interesting to compare patients with the neurodegenerative disease, HD, with others who were suffering brain injury, both those in a clinically stable condition with severe chronic brain injury, mainly caused by a traumatic injury, and those immediately after acute stroke.

Methods were developed and validated for the determination of the concentrations of tryptophan, kynurenine, KYNA, ANA, 3HKYN and 3HANA in plasma or serum. The method for 3HKYN had low sensitivity.

This project has demonstrated increased IDO activity, measured by an increase in the K:T ratio, in the blood of HD, chronic brain injury and stroke patients. The increased IDO activity indicates activation of the kynurenine pathway, and alterations in the concentrations of kynurenines have been observed for all three disorders.

After oral tryptophan depletion, the differences in the concentrations of kynurenines observed at baseline, between HD or chronic brain injury patients and controls, were

maintained. Tryptophan loading increased the levels of all the kynurenine metabolites and there were fewer differences between patients and controls. Tryptophan loading may be detrimental for healthy subjects as it was shown to induce lipid peroxidation and inflammation.

There were no changes in the levels of any of the kynurenines in the 14 days following an acute stroke. Patients who died during the study or immediately afterwards (within 3 weeks of the stroke) had high levels of KYNA in their blood.

The increase in IDO activity was accompanied by an increase in the inflammatory marker, neopterin, for each disorder, suggesting that IDO may be activated by inflammatory mediators. Neopterin was significantly increased in the chronic disorders (HD and chronic brain injury) showing that inflammation persists several years after the original brain injury and that inflammation is involved in late stage HD. The high lipid peroxidation for the HD and chronic brain injury patients suggested that high levels of oxidative stress were contributing to progressive neurodegeneration in HD and to continuing brain damage in chronic brain injury.

In the HD and chronic brain injury patients, there was low KAT activity, suggesting a lack of production of the neuroprotectant, KYNA, which may be triggered by chronic inflammation and increased IDO activity.

The most interesting and novel result from this project was the low levels of 3HANA determined in all three disorders, and the associated low levels of 3HKYN in the HD and chronic brain injury patients. Unfortunately, 3HKYN was not measured in the stroke patients. The increased levels of ANA and the 3HANA: ANA ratio may also be of key importance in stroke.

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Appendix

Methods carried out by others

Quinolinic Acid by GC-MS (Dr J. Christofides and Dr M. Egerton, West Park Biochemistry Laboratories, Epsom General Hospital, Epsom, Surrey, UK)

Levels of quinolinic acid were measured by GC-MS of the t-butyldimethylsilyl derivative, after sample clean up using SPE. 1ml plasma was mixed with dipicolinic acid (the internal standard) and acetate buffer at pH 2.0-2.2. SPE sample cleanup was with a Waters SepPak C18 cartridge, with quinolinic acid being eluted in 2% ammonia in methanol. Prazepam was added to the sample eluate as an extra recovery standard and the mixture was dried. Derivatisation was achieved by adding *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide and 1% tert-butyldimethylchlorosilane and incubating for 2h at 90°C. The derivatised extract was analysed by GC-MS using a Thermo GC8000 and MD800 quadrupole mass detector. 1µl sample was injected using splitless injection at 190°C on to a 30m x 0.2mm RTX5 GC column. The GC oven temperature was increased from 120°C to 230°C over 5 minutes followed by a rise to 260°C over 16 minutes. The detector, using electron impact at 70eV, was set to maximum voltage monitoring ions at mass/charge ratio (m/z) of 338.1 and 380.1 with a dwell time of 0.3 seconds.

Standard clinical assays – ESR and CRP (Haematology and Immunology Laboratories, Epsom General Hospital, Epsom, Surrey, UK)

Erythrocyte sedimentation rate (ESR) was determined as a marker of inflammation. ESR is measured as the distance a column of anticoagulated blood will fall under gravity in 1 hour. Red blood cells have a surface charge which prevents them from coming too close to each other. With infection or inflammation, there is increased production of fibrinogen and other large charged molecules that allow the red blood cells to come closer to each other and fall faster in an ESR test. ESR was measured by a StaRRsed Automated ESR machine (RR Mechatronics) which utilises the method recommended by the International Council for Standardization in Haematology (1933).

C-reactive protein (CRP) is another measure of acute inflammation. In blood, levels of CRP, an acute phase reactant, rise dramatically with inflammation. CRP was measured using a Behring Turbitimer.

Neopterin assay (Dr C.M. Forrest and Ms L. Oxford, NABS, IBLS, University of Glasgow, Glasgow, UK)

Neopterin levels were measured in 10µl aliquots of serum using an enzyme linked immunosorbent assay (ELISA) kit (Immunobiological Laboratories, Germany). The assay was a competitive ELISA where a peroxidase-conjugated neopterin competed with sample neopterin for a fixed number of antibody binding sites. Peroxidase- conjugated neopterin-antibody complexes bind to the wells of the plate, coated with another antibody. The plate is incubated with shaking to enable the reactions to occur. Unbound peroxidase-conjugated neopterin is removed by washing using a plate washer. Following incubation with substrate solution, colour developed depending on the level of bound peroxidase complexes. The optical density is inversely proportional to the neopterin concentration of the sample. The optical density was read at 450nm using a plate reader. Neopterin standards of known concentrations were assayed together with the samples, and were used to prepare a calibration curve for quantifying the samples. All samples were analysed in duplicate.

Lipid peroxidation products (Dr C.M. Forrest and Ms L. Oxford, NABS, IBLS, University of Glasgow, Glasgow, UK)

The concentrations of the lipid peroxidation products malondialdehyde and 4-hydroxyalkenals in serum samples were measured using a Bioxytech LPO-586 colorimetric assay (Biostat). Lipid peroxidation products in 100µl of serum, stabilised with BHT, were reacted with *N*-methyl-2-phenylindole in methanesulfonic acid at 45ºC to form a stable chromogenic indolic dimer. The reaction mixture was centrifuged in Anopore filter tubes to clarify the solutions. The absorbance was measured spectrophotometrically at 595nm and malondialdehyde and 4-hydroxyalkenals quantified from a calibration curve of 4-hydroxynonenal standards. All samples were tested in duplicate.