BIOCHEMICAL ASPECTS OF TOURETTE'S SYNDROME

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Dedicated to my wife Tracey

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Biochemical Aspects of Tourette's Syndrome

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Summary

Kynurenine (KYN) is the first stable metabolite of the kynurenine pathway, the major route of tryptophan (TRP) metabolism. In the liver, cortisol-inducible tryptophan-2,3-dioxygenase (TDO) is the first enzyme and rate limiting step. In extrahepatic tissues, it is superceded by indoleamine-2,3-dioxygenase (IDO), an enzyme with a wider substrate specificity. Earlier work in this research group has found substantial elevations in plasma KYN in fasting Tourette's Syndrome (TS) patients with normal TRP and neopterin. The aim of our initial pilot study was to confirm this increase in KYN in fasting human TS patients compared with normal controls, and to see how changes in diet may influence certain kynurenine pathway variables. However, we failed to detect a change in plasma KYN, TRP, kynurenic acid (KYNA), neopterin or cortisol between the fasting TS and control groups. Moreover, none of the variables was affected by dietary status, and thus candidates selected for the larger cross-sectional study were permitted to eat and drink freely on the day that blood samples were submitted, but were requested to avoid products containing caffeine, aspirin or nicotine.

In the cross-sectional study, TS patients exhibited significantly higher plasma KYN concentrations than controls, although the magnitude of the change was much smaller than originally found. This may be due to differences in detection procedure and the seasonal fluctuation of some biochemical variables, notably cortisol. The generalised increase in neopterin in the TS subject group, suggests a difference in the activity of cytokine-inducible IDO as a likely source for this elevated KYN. Other kynurenine pathway metabolites, specifically TRP, 3-hydroxykynurenine (HKY), 3-hydroxyanthranilic acid (HAA) and KYNA were unchanged. In view of recent speculation of the potential therapeutic effects of nicotine in TS, the lower KYN concentrations observed in TS smokers, compared with non-smoking TS patients, was another interesting finding.

Tic-like movements, such as head-shakes (HS), which occur in rodents both spontaneously and following diverse drug treatments, closely resemble tic behaviours in humans. The animal tic model was used to examine what effects nicotine may have on shaking behaviours, and on selected TRP metabolites. Acute systemic administration of nicotine to mice, produced a dose-dependent reduction in HS frequency (induced by the 5-$HT_{2A/2C}$ agonist DOI), which appeared to be mediated via central nicotinic cholinergic receptors, since mecamylamine pretreatment abolished this effect. Conversely, twice daily subcutaneous injections of nicotine for 7 days, led to an increase in spontaneous and DOI-induced HS. Chronic nicotine also caused a significant elevation in plasma and whole brain KYN concentrations, but plasma TRP, HKY, HAA and KYNA were unaltered. In addition, no change in brain 5-HT or 5-HIAA concentrations, or 5-HT turnover, was found. Despite contrasting results from human and animal studies, a role for nicotine in the mediation of tic-like movements is indicated.

The relevance of the kynurenine pathway to TS and the potential role played by nicotine in modifying tic-like behaviours is discussed.

Key words:

Kynurenine, tryptophan-2,3-dioxygenase, indoleamine-2,3-dioxygenase, Tourette's Syndrome, neopterin, nicotine, tic-like movements, head-shakes.

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ABBREVIATIONS

3-HAO	3-hydroxyanthranilic acid oxygenase
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
5-MeODMT	5-methoxy-N,N-dimethyltryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino) tetralin
AA	anthranilic acid
ADHD	attention deficit hyperactivity disorder
ASO	antistreptolysin O
BDI	Beck depression inventory
CNS	central nervous system
CSF	cerebrospinal fluid
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DOPAC	3,4-dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECD	electrochemical detection
EDTA	disodium ethylene diamine tetraacetic acid
GABA	γ-aminobutyric acid
GTP	guanosine triphosphate
HAA	3-hydroxyanthranilic acid
HKY	3-hydroxykynurenine
HPLC	high performance liquid chromatography
HS	head-shake
HVA	homovanillic acid
IDO	indoleamine-2,3-dioxygenase
i.p.	intraperitoneal
KAT	kynurenine aminotransferase
KYN	kynurenine
KYNA	kynurenic acid
L-DOPA	*L*-dihydroxyphenylalanine

LNAA	large neutral amino acids
LOI	Leyton obsessional inventory
MHC	major histocompatibility complex
MHPG	3-methoxy-4-hydroxy-phenethyleneglycol
MRI	magnetic resonance imaging
MTE	normetanephrine
NAD	nicotinamide adenine dinucleotide
OCB	obsessive-compulsive behaviour
OCD	obsessive-compulsive disorder
pCPA	*p*-chlorophenylalanine
PET	positron emission tomography
QPRT	quinolinic acid phosphoribosyl transferase
QUIN	quinolinic acid
s.c.	subcutaneous
SEM	standard error of the mean
SIB	self-injurious behaviour
STAI	Spielberger state-trait anxiety index
TDO	tryptophan-2,3-dioxygenase
TRH	thyrotropin releasing hormone
TRP	tryptophan
TS	Tourette's syndrome
UV	ultraviolet
WHO	World Health Organisation
WDS	wet-dog-shake
XANA	xanthurenic acid
YGTSS	Yale Global Tourette severity score

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1. General

Tourette's syndrome (TS) is a unique eponym bestowed on the disease, after its symptoms were accurately described by the French physician Gilles de la Tourette back in 1885. With particular emphasis on the existence of multiple tics, coprolalia (involuntary inappropriate uttering of obscenities) and echolalia (imitation of sounds or words), he astutely maintained that TS was a neurological disorder, as opposed to a form of insanity or psychiatric illness. Although the hereditary nature of the condition is well established, researchers have not yet succeeded in identifying and locating the specific gene(s), or markers linked to the gene(s). Moreover, the underlying neurochemical abnormality of TS remains poorly understood, with drugs currently used for its treatment offering considerable disadvantages. Following reports of possible differences in the kynurenine metabolic pathway in TS, which acts as the major route of tryptophan (TRP) metabolism, the studies carried out in this thesis have attempted to focus on the likely mechanism of such changes. The simultaneous collection of clinical, psychopathological, biochemical and other data, enabled us to evaluate the relationships between a wide range of factors having potential relevance to TS and the kynurenine pathway. Experiments were also carried out to examine the capacity of nicotine to modify tic-like behaviours in animal models.

2. Differential diagnosis of tic disorders

Tics are sudden, rapid, recurring, non-rhythmic, stereotyped motor movements or vocalisations. Tics are not truly involuntary, as they can typically be suppressed for brief periods of time, and may be accompanied by a premonitory sensory urge (Bliss, 1980; Leckman *et al.*, 1993). Persons with tics often describe the need to perform the action as an irresistible compulsion, with some sufferers being able to identify a sensory precursor (Bliss *et al.*, 1980). The frequency and intensity of the tics is typically increased in situations of anxiety, anger, emotional stress, excitement, boredom and fatigue (Robertson, 1989; Singer and Walkup, 1991), and tend to be

less prominent during sleep, periods of relaxation or concentration and with ingestion of alcohol (Robertson *et al.*, 1988; Robertson, 1989; Singer and Walkup, 1991).

The clinical criteria widely accepted for the diagnosis of TS are laid out in the Diagnostic and Statistical Manual of Mental Disorders (Fourth edition) (A.P.A, 1995), which is generally written in the abbreviated form DSM-IV. These criteria are:

A. Both multiple motor and one or more vocal tics have been present at some time during the illness, although not necessarily concurrently.

B. The tics occur many times a day (usually in bouts), nearly every day or intermittently throughout a period of more than one year, and during that period there was never a tic-free period of more than 3 consecutive months.

C. The disturbance causes marked distress or significant impairment in social, occupational, or other important areas of functioning.

D. Onset before age 18 years.

E. The disturbance is not due to the direct physiological effects of a substance (e.g. stimulants) or a general medical condition (e.g. Huntington's chorea or post-viral encephalitis).

The manual also lays down criteria for the classification of Chronic Motor or Vocal Tic Disorder and Transient Tic Disorder. The distinguishing criterion for Chronic Motor or Vocal Tic Disorder states that that either motor or vocal tics, but not both, must have been present at some time during the illness. Transient Tic Disorder, on the other hand, differs from TS by the duration of its symptoms, which must be present for at least four weeks, but no longer than twelve consecutive months. Furthermore, there must be no history of Tourette's or Chronic Motor or Vocal Tic Disorder.

Certain other disorders of the central nervous system (for example Huntington's chorea, Sydenham's chorea or encephalitis) may present symptoms of a similar nature to tic disorders. In addition, numerous reports of tic-like symptoms have been presented in the literature following long and short-term use of neuroleptics (Klawans *et al.*, 1978; Mueller and Aminoff, 1982; Lal and Alansari, 1986) and after treatment with psychostimulants such as methylphenidate, dextroamphetamine and pemoline (Golden, 1974; Denckla *et al.*, 1976; Lowe *et al.*, 1982), which are frequently used

to treat children suffering from Attention Deficit Hyperactivity Disorder (ADHD). In these latter cases, the precise origin of the tics can be particularly difficult to establish, owing to the considerable comorbidity of ADHD in subjects with tic conditions. Hence, a thorough investigation into the patients medical history is necessary to ensure the correct diagnosis is made.

3. Clinical Features

The age of onset of TS commonly ranges from 2 to 15 years, but by the age of 11 years old symptoms have appeared in 96% of patients (Robertson, 1989). The mean age of onset is 7 years old, and the initial symptoms most frequently exhibited are tics involving the eyes (in particular eye-blinking), which occur in 39-59% of cases, although head and facial tics are other common presenting tics (Robertson, 1989). Examples of facial tics include: nose-twitching, stretching of the mouth in any direction, pursing the lips, as well as combinations of all of these (Comings and Comings, 1985).

Vocal tics typically appear later than motor tics, emerging at a mean age of 11 years old. The most frequent vocalisations are repeated throat-clearing (Comings and Comings, 1985; Regeur *et al.*, 1986), but other examples include grunting, coughing, barking, snorting, explosive noises, word accentuation, humming, hissing, clicking, colloquial emotional exclamations, low and high-pitched noises, inarticulate sounds and coprolalia (Robertson, 1989). In one cohort of 90 TS patients, the mean cumulative number of vocalisations was reported to be 4.8 per individual (Robertson *et al.*, 1988).

Comings and Comings (1985) found that the most characteristic tic of the head and neck was the 'hair-out-of-the-eye' tic. Regeur *et al.* (1986) reported licking as a motor tic in 20% of TS patients, and spitting was exhibited by 9% of subjects in another study (Robertson *et al.*, 1988). Furthermore, cumulative lifetime motor tics have been estimated for the face (94-97%), head, neck and shoulder (51-81%), legs (40-55%) and body (41-54%) (Robertson, 1989).

Some TS patients also perform complicated movements, otherwise known as complex tics, due to the coordinated and seemingly purposeful nature of the actions (Kurlan, 1989). The most frequently exhibited complicated movements include

touching (38-61%), hitting or striking (35-63%), jumping (28%), smelling of hands (12%), smelling of objects (11%), stamping (9%), squatting (7%) and various complexities of gait, such as retracing steps (5%), twirling (5%) and deep knee bends (4%) (Robertson, 1989).

Coprolalia is not a diagnostic criterion for TS, but is undoubtedly one of the most socially distressing symptoms. Its mean age of onset is commonly between 13 and 14.5 years (Robertson, 1989), and more recent estimates of the prevalence of coprolalia in American and U.K. cohorts are 33-37% of TS subjects (Comings and Comings, 1985; Shapiro and Shapiro, 1986; Robertson *et al.*, 1988). The presence of copropraxia (involuntary and inappropriate obscene gestures) has been reported in 18-21% of UK patients, the commonest gesture being the palm-backed 'V sign' (Lees *et al.*, 1984; Robertson *et al.*, 1988). Interestingly, Butler (1984) noted an apparent infrequency of coprophenomena in certain TS patients from middle class and strict religious backgrounds.

Motor and vocal tics are affected by a multitude of environmental factors and components of lifestyle, which may help to explain the waxing and waning nature of the symptoms (refer to previous section). Furthermore, TS is characteristically punctuated by the emergence of new tics and disappearance of older ones during the patients life.

The prognosis for individual patients is difficult to predict, although complete remission from symptoms is uncommon, with one report indicating spontaneous remission of symptoms in just 3.3% of patients (Abuzzahab and Anderson, 1973). For many TS sufferers, adulthood marks an improvement in the number and severity of tics, plus reduced social impairment and new ways of coping with various aspects of the disorder (Cohen and Leckman, 1994). Judging the natural progression of this condition is further complicated by the coexistence of other conditions and psychopathologies (see: Associated Features).

4. Epidemiology and Population studies

The currently accepted figure for the prevalence of TS in the population is 0.5 per 1000 (Bruun, 1984), but Robertson (1989) suggested that this quotient may prove to be something of an underestimate. Indeed, since milder manifestations of TS may

occur, where the symptoms are non-impairing, many cases might not be brought to the attention of the medical profession, which could account for doubts about the exact prevalence of TS (Kurlan *et al.*, 1987).

TS is found in all cultures and racial groups (Robertson, 1989), although an under-reporting of cases in some countries may explain the variation in prevalence estimations in different geographical areas. This is suitably highlighted by a survey carried out in New Zealand by Robertson *et al.* (1994), in which 40 probable cases of TS were identified, whereas only 3 cases had been documented prior to this study.

There seems to be wide agreement that TS occurs three to four times more commonly in males than in females (Corbett *et al.*, 1969; Moldofsky *et al.*, 1974; Burd *et al.*, 1986; Regeur *et al.*, 1986; Robertson *et al.*, 1988). The condition exists in all social classes (Moldofsky *et al.*, 1974; Nee *et al.*, 1980), but Nee and colleagues (1980) found a higher percentage in the lower social classes. Furthermore, it was reported that 61-63% of TS patients failed to reach the social class attained by their parents (Asam, 1982; Robertson *et al.*, 1988).

5. Associated Features

Even dating back to the earliest descriptions of TS, a frequent presence of comorbid psychopathologies has been noted in TS patients, and evidence from genetic studies is suggestive of an intimate link between TS and obsessive-compulsive disorder (OCD). Despite the recognition of a variety of other concomitant behavioural conditions, such as attention deficit hyperactivity disorder (ADHD), learning disorders and self-injurious behaviour (SIB), there is little grounds to support such an association between TS and these additional disorders. Nonetheless, it is interesting that the symptoms of these coexisting disorders are often the reason for which the patient is initally referred to a physician (Robertson, 1989), some of which will be described in the following section.

Obsessive-compulsive disorder:

Obsessive-compulsive symptoms are characterised by the presence of recurrent, stereotyped behaviours (which the individual often recognises as being irrational or excessive) and/or thoughts that intrude into consciousness or actions. When

symptoms result in significant distress or impairment, it is classified as a 'disorder'. Examples of compulsive symptoms are ordering and arranging habits, touching one's body, repeated hand washing, checking rituals (such as door locks and windows), frequent counting and rituals for decontaminating objects or body parts (Singer and Walkup, 1991). Common obsessive symptoms include fears or images of loved ones being harmed, of contamination with dirt or germs, fear of being responsible for the misfortune of others, or recurring doubt that the individual has actually performed a task (Singer and Walkup, 1991).

To distinguish compulsions from tics, DSM-IV (A.P.A, 1995) uses the criterion of purpose: in the former case, "the person feels driven to perform the compulsion to reduce the stress that accompanies an obsession or to prevent some dreaded event or situation," whereas tics are described as involuntary, are "typically less complex and are not aimed at neutralising the anxiety resulting from an obsession."

The high incidence of OCD in TS patients has been confirmed in several studies (Comings and Comings, 1985; Montgomery *et al.*, 1982; Pitman *et al.*, 1987; Robertson *et al.*, 1988), and genetic studies indicate that OCD in some individuals may be an integral part of TS, representing a different phenotypic expression of the same genotype (see: Genetics). The exact prevalence of OCD in patients suffering from TS is uncertain, owing largely to variations in diagnostic criteria, interview techniques, and structure of questionnaires used for the assessment of symptoms. However, the estimations provided by two separate studies have provided comparable data: in one sample of 32 TS patients Pauls *et al.* (1986b) reported a frequency for comorbidity of TS and OCD of 50%, whereas the corresponding figure calculated by Pitman *et al.* (1987) was 63% (10 out of 16 TS patients).

Frankel *et al.* (1986) noted an age-related difference in obsessive-compulsive symptoms in TS patients. Younger subjects typically had impulse control problems, whereas older TS patients were more concerned with checking, arranging and fear of contamination. Examination of the symptoms most commonly experienced in OCD by subjects with comorbid TS, compared with 'pure' OCD, has also revealed some striking differences. Panic and phobic disorders occur frequently in patients with OCD alone, but not in TS cohorts. In contrast, patients with TS are more likely to exhibit touching compulsions, arithmomania (counting rituals or obsessions with

numbers) and 'evening-up' behaviours designed to maintain a sense of right-left bodily symmetry (Frankel *et al.*, 1986; Pitman *et al.*, 1987; Caine *et al*, 1988).
Nevertheless, there are some clinical similarities between OCD and TS, such as chronic waxing and waning course, exacerbation with stress and the typical lifelong duration of both disease states (Frankel *et al.*, 1986; Singer and Walkup, 1991).

Attention-deficit hyperactivity disorder:

Attention-deficit hyperactivity disorder (ADHD) has a number of characteristic elements comprising of short attention span, restlessness, poor concentration and diminished impulse control. In affected individuals, the symptoms of ADHD are generally recognised to precede the progression to TS (Singer and Walkup, 1991). The prevalence and spectrum of symptoms exhibited is also subject to variation; attention-deficit disorder (ADD) was found in 13% of one small cohort of TS patients (Lieh Mak *et al.*, 1982), but in a second larger study ADD associated with hyperactivity was shown by 54% of the sample group (Comings and Comings, 1985), whereas hyperactivity alone occurred in 67% of TS subjects assessed by Moldofsky *et al.* (1974).
Although a higher than normal incidence of ADHD among TS cohorts is accepted, the association between these conditions is more controversial. Comings and Comings (1990) hypothesise that ADHD (and learning disorders) form an integral part of the expression of the TS gene(s). In contrast, other research groups have found no evidence of a genetic association between TS and ADHD (Pauls *et al.* 1986a), or indeed between TS and ADD, learning disabilities, speech disorders or stuttering (Pauls *et al.*, 1993).
A further cause for concern has been the propensity for stimulant medication (which is sometimes used to treat ADHD) to actually provoke tics (Golden, 1974; Lowe *et al.*, 1982). As a result, methylphenidate, dextroamphetamine and pemoline have been investigated for their tendency to provoke or exacerbate tics in susceptible individuals. Despite evidence indicating that tic symptoms are worsened by this group of drugs (Erenberg *et al.*, 1985; Price *et al.*, 1986), psychostimulants are not considered as the primary cause for tics, since ADHD symptoms typically precede the

appearance of tics. Genetic studies appear to offer additional confirmation of this view (Price *et al.*, 1986).

Learning difficulties:

There is evidence that children with TS may experience difficulties in the classroom, and Erenberg *et al.* (1986) reported learning problems in 36% of 200 children with TS. These problems may be directly or indirectly related to the tic symptoms, for example the tics can disrupt other classmates, interfere with handwriting or affect their participation in class discussions (Singer and Walkup, 1991). Additional problems can similarly be created by the comorbid behavioural disturbances, such as OCD and ADHD, which can affect attention, concentration and task completion. Of course the possible adverse effects of medication used in the treatment of patients can also present problems, and this is one of the many factors which needs to be considered by physicians.

Self-injurious behaviour:

Self-injurious behaviour (SIB) is self-inflicted deliberate harm to one's own body, and shows a high prevalence in TS patients, with one study reporting that 33% of 90 TS subjects had admitted to SIB (Robertson *et al.*, 1989). The types of behaviour commonly encountered ranged from self-slapping and scratching parts of the body, to poking sharp objects into the body and head-banging (resulting in cavum septum pellucidum cavities). Self-injury was correlated with severity of TS symptoms and associated psychopathologies, but showed no correlation with intelligence (Robertson *et al.*, 1989).

Other associated features:

There are a number of other psychopathologies and behavioural disturbances which display an unusually high level of incidence in TS patients, including depressive illness, aggression and sleep abnormalities (Robertson *et al.*, 1988). Robertson and colleagues found that 85% of subjects encountered disorders of sleep, with night terrors and insomnia most commonly described, and these appeared unrelated to any medication being taken.

Other disabling features associated with TS comprise anti-social behaviour, inappropriate sexual activity, exhibitionism, discipline problems, anger and violence (reviewed in: Robertson, 1989). The diverse range of behavioural problems frequently encountered by TS patients, however, seems more closely related to the effects of suffering from a stigmatising disorder, or the accompanying ADHD and obsessive-compulsive symptoms, rather than a direct effect of the tics (Singer and Walkup, 1991).

6. Genetics

In his original description of TS, Gilles de la Tourette (1885) commented on the hereditary nature of the disease. Interest resurfaced following reports of multiple affected members within a family (Erickson and Persson, 1969; Friel, 1973; Lucas, 1973), and due to the development of improved techniques to aid research in genetics (for example segregation and linkage analysis).

Twin studies have provided the most compelling evidence for a genetic component to TS (Jenkins *et al.*, 1983; Price *et al.*, 1985; Goetz and Tanner, 1990). In one study involving 43 pairs of same-sex twins, in which at least one co-twin had TS, the concordance for TS in monozygotic and dizygotic twins was 53% and 8% respectively (Price *et al.*, 1985). When the diagnostic criteria were expanded to include chronic tics in twins, concordance rates rose to 77% in monozygotic pairs and and 23% in dizygotic pairs. The incomplete concordance for monozygotic twins suggested that other non-genetic factors were also involved (see: Influence of environmental factors on TS expression). However, a follow-up study, which re-evaluated 19 of the original 30 monozygotic twins assessed by Price and colleagues, claimed a concordance rate of 89% for TS and 100% for TS or chronic motor tics (unpublished data, refer to: Singer and Walkup, 1991), which suggested that chronic motor tic disorder is genetically related to TS. Kidd *et al.* (1980) used segregation analysis to confirm the genetic relationship between chronic motor tics and TS, as well as demonstrating that the sex difference in the prevalence of TS is real, since multiple tic conditions occurred more frequently in the male relatives of the TS probands than in female relatives.

Studies indicate that a single major gene contributes to expression of TS, and that females (the less prevalent sex in TS) have a higher genetic loading for the disorder (Kidd *et al.*, 1980; Baron *et al.*, 1981). In their study involving 127 TS patients (plus relatives), Baron et al. (1981) highlighted the higher proportion of affected relatives of female probands compared with relatives of male probands. Furthermore, the predicted population prevalence of TS and chronic tics for the higher threshold female category was lower than that predicted for the lower threshold disorder, *i.e.* affected males (0.8% vs. 2.3%)(Baron *et al.*, 1981). Pauls and Leckman (1986) examined the mode of transmission of TS, and broadened the phenotype to include both chronic motor tics and OCD, because of previous data which suggested that chronic tics and OCD may be aetiologically related (Pauls *et al.*, 1986b). The results of the study were consistent with an autosomal dominant mode of transmission for TS and associated behaviours, and suggested that there may be a sex difference not only in the frequency of TS, but also in the frequency of expression of related behaviours, such as OCD (Pauls and Leckman, 1986). More recent investigations employing similar diagnostic criteria supported the theory of an autosomal dominant gene with high penetrance (Robertson and Gourdie, 1990; Eapen *et al.*, 1993).

The widely accepted hypothesis of autosomal dominant transmission of TS was challenged by Comings *et al.* (1989), who proposed that inheritance in TS may be better described as semi-dominant, semi-recessive, but this suggestion was based on the highly contentious assumption that several other disorders (including ADHD, alcoholism, drug abuse and panic disorder) may also be gentically related to TS. Among the arguements to counter the controversial ideas of Comings (see: Comings, 1990b), are that the stigmatising effects of TS may be partly responsible for a greater prevalence of certain conditions (such as alcohol and drug abuse) in TS patients.

Evidence implicating an abnormality in dopaminergic neurotransmission in TS (see: Aetiology of TS) has influenced many researchers to employ genes for dopamine receptors as candidates for linkage studies. Gelernter *et al.* (1990) found no evidence for linkage of TS to the dopamine D_2 receptor, or to the D_1 receptor subtype (Gelernter *et al.*, 1993). In contrast, Comings *et al.* (1991) examined the dopamine D_2 receptor locus as a phenotypic expression of a number of psychiatric, behavioural and neurological disorders (including TS, ADHD, autism and alcohol or drug

addiction/abuse), and reported that the prevalence of the A1 allele of the dopamine D_2 receptor gene was significantly increased in patients with TS, ADHD, alcoholism, autism and post-traumatic stress disorder. Comings and coworkers speculated that the A1 allele of the D_2 receptor gene is not the primary aetiological cause of these disorders, but instead acts as a modifier gene, and may simply cause an increase in symptom expression in individuals who possess the primary gene for the condition. An association between TS and the dopamine D_3 receptor gene has also been reported (Comings *et al.*, 1993), although this claim was disputed by Hebebrand *et al.* (1993).

More recently, Lam *et al.*, (1996) found a serotonin receptor gene (5-HT_{1A}) variant in a single TS patient (out of a sample size of 21), but Brett *et al.* (1995) eliminated the 5-HT_{1A} receptor or tryptophan oxygenase genes from having a role in the pathophysiolgy of TS.

Linkage studies have been extensively used in an effort to locate the gene(s) for the vulnerability to TS, and such methods have presently excluded approximately 85% of the genome (Brett *et al.*, 1995). Should the entire genome be excluded, then the underlying assumptions frequently employed may need to be reassessed, for example the assumption of genetic homogeneity (i.e. the disorder is caused by the same single gene locus in each family), and secondly that TS and chronic multiple tics are alternate expressions of the same underlying genetic vulnerability.

7. Influence of environmental factors on expression of TS

In spite of disagreement between some groups about the likely mode of transmission of TS, the hereditary nature of the disorder is widely accepted. However, the incomplete concordance for TS and other tic disorders exhibited by monozygotic twin pairs (Price *et al.*, 1985; Hyde *et al.*, 1992), has prompted consideration of how certain non-genetic factors may figure in the phenotypic expression of TS. Hyde *et al.* (1992) also pointed to the striking variation in the severity of TS symptoms in affected pairs, and concluded that since these individuals are genetically identical, the observed differences in concordance and severity cannot be due to genetic influences alone. After analysing a sample of their own patients, Shapiro and Shapiro (1982) found no association between tic severity and certain pre-natal factors, including birth

weight, birth order, perinatal complications, history of parental abortions, age of parents at time of birth, patient medical history and family medical history. In contrast, an investigation of discordant monozygotic twins, showed that the more severely affected individual generally had a lower birth weight (Leckman *et al.*, 1987). Moreover, Hyde *et al.* (1992) reported that in 12 out of 13 cases, the magnitude of intrapair birth weight variance was directly proportional to the observed difference in tic severity, indicated by tic score. Furthermore, there was no correlation between tic severity and post-natal medical events (such as childhood infections, surgery and birth complications), suggesting that events *in utero* were more likely to affect the phenotypic expression of TS. Additional pre-natal factors, which have been subject to scrutiny comprise maternal stress during pregnancy and gender of child (Leckman *et al.*, 1990; Leckman and Peterson, 1993). The greater prevalence of TS in males (see: Epidemiology and population studies) and its frequent pre-pubertal onset, prompted speculation that exposure of the developing male brain to elevated levels of testosterone and other androgenic steroids early in foetal life, may have a significant bearing on the later emergence and prognosis of TS and related disease states (Leckman and Peterson, 1993). This hypothesis is supported by genetic evidence that although TS is more common in males, it does not appear to be transmitted as a sex-linked trait (Kidd *et al.*, 1980). In addition, reports indicate that exposure to anabolic steroids may exacerbate tics, whereas an amelioration of tic symptoms has been observed following treatment with the androgen receptor blocking drug flutamide (Peterson *et al.*, 1992; Peterson *et al.*, 1994). Correlation between levels of maternal emotional stress during pregnancy and tic severity in the offspring have also been documented (Leckman *et al.*, 1990), although the results were interpreted with caution owing to the problems of interviewing mothers about events often occurring several years previously. The authors recognised the potential for recall bias to affect the results, since those mothers with children more severely affected with TS symptoms at the time of interview, may inadvertently remember the events surrounding the childs birth as being more problematic. The waxing and waning nature of TS symptoms further complicate the validity of these deductions. However, in support of a possible association between maternal stress during pregnancy and severity of tic disorders,

Leckman and Peterson (1993) referred to studies where adult rats previously exposed (*in utero*) to a regimen of pre-natal stresses, demonstrated an increased vulnerability to stressful stimuli, as well as region-specific differences in biogenic amine concentrations (particularly dopamine), and altered cerebral assymmetries (Fride and Wienstock, 1988). Other related studies have implicated changes to the development of noradrenergic, serotonergic and opioid systems of offspring, and increased plasma adrenocorticotrophic hormone (ACTH) and corticosterone in pre-natally stressed rats (Rosengarten and Friedhoff, 1979; Moon, 1984; Leckman and Peterson, 1993).

8. Neuroanatomic localisation

The precise neuroanatomic localisation of the dysfunction in TS remains unclear, but much of the early evidence favouring the basal ganglia as the primary site, stemmed from its established role in other movement disorders, such as Parkinson's disease and Huntington's chorea. Volumetric magnetic resonance imaging (MRI) studies comparing TS patients and controls have proved supportive to this hypothesis (Peterson *et al.*, 1993; Singer *et al.*, 1993). Singer *et al.* (1993) reported that although no significant variation was found in the size of the right or left caudate, putamen, globus pallidus or ventricles between TS and control groups, children with TS showed assymmetries of the putamen and lenticular region that differed from those in controls - in particular, a shift in the left-sided predominance seen in controls. In an adult study carried out by Peterson *et al.* (1993), a significantly smaller mean left lenticular volume was exhibited by the TS group. Positron emission tomography (PET) studies with ^{18}F-labelled fluoro-deoxy-D-glucose showed that glucose utilisation in the basal ganglia of TS subjects averaged 16% above control levels, but differences were also apparent in the frontal and temporal lobes bilaterally (Chase *et al.*, 1984).

Bonnet (1982) approached the question of the primary anatomical locus of TS by focussing on neurochemistry and the anatomical substrates of vocalisation and various movements, including blinking and tics. In his conclusion he suggested the biochemical structure of the limbic forebrain structures, particularly the anterior cingulate cortex, and their interrelationship with the specific nuclei of the tegmentum, renders the cingulum the possible anatomical site for TS.

9. Aetiology of TS

Despite extensive research into the aetiology of TS, the precise biochemical basis for the disorder has yet to be elucidated, although an abnormality of several neurotransmitter systems remains a distinct likelihood. Consequently, pharmacological agents presently used in the treatment of TS, at best, provide symptom relief rather than a cure for the underlying condition.

A defect in the dopaminergic system in the pathophysiology of TS has been implicated by the effectiveness of neuroleptics (primarily haloperidol and pimozide) in ameliorating many of the symptoms of the disorder (Shapiro and Shapiro, 1981; Shapiro and Shapiro, 1982; Shapiro *et al.*, 1989; Cohen *et al.*, 1992), and reports that stimulants (such as pemoline and methylphenidate) may exacerbate motor and phonic tics (Erenberg *et al.*, 1985; Lowe *et al.*, 1982; Price *et al.*, 1986). Furthermore, some TS patients have shown symptom improvement following use of agents that prevent accumulation of dopamine in presynaptic storage vesicles, for example tetrabenazine (Jankovic *et al.*, 1984), and with drugs that block dopamine synthesis, such as α-methyl-*p*-tyrosine (Sweet *et al.*, 1974). Biochemical studies have demonstrated a decrease in the CSF levels of the dopamine metabolite homovanillic acid (HVA) in TS subjects, which was restored after administration of the antidopaminergic agent chlorpromazine, and accompanied by an improvement in clinical symptoms (Cohen *et al.*, 1978; Singer *et al.*, 1982). Additional evidence for involvement of dopaminergic systems in the aetiology of TS has been provided by postmortem brain studies, where an elevation in the number of dopamine uptake carrier sites has been found (Singer *et al.*, 1991), possibly indicative of excess dopamine release and receptor activation. However, since the mean levels of dopamine and its metabolites HVA and 3,4-dihydroxyphenylacetic acid (DOPAC) in the caudate and putamen of the brains were normal, and the density of dopamine D_1 and D_2 receptor sites was unchanged between TS and control samples, Singer and colleagues (1991) theorised that dopamine hyperinnervation was a likely causative factor in TS. PET studies relating to dopamine receptor alterations in TS have provided conflicting data. Wong *et al.* (1989) found a significantly greater number of dopamine D_2 receptors in the striatum of TS patients compared with controls,

although other research groups reported no difference (Brooks *et al.*, 1992; Singer *et al.*, 1992). Genetics investigations concentrating on the involvement of dopamine receptor subtypes in the aetiology of TS has further fuelled disagreement (see: Genetics).

The efficacy of the α_2-adrenergic agonist clonidine in the treatment of TS (Singer *et al.*, 1985-86; Leckman *et al.*, 1991; Cohen *et al.*, 1992) may indicate a role for noradrenergic mechanisms, although the effectiveness of clonidine has been questioned (Messiha, 1988; Goetz, 1992). A possible noradrenergic deficit has been suggested by decreased urinary excretion of noradrenaline metabolites 3-methoxy-4-hydroxy-phenethylene glycol (MHPG) and normetanephrine (MTE) in TS patients (Ang *et al.*, 1982; Baker *et al.*, 1991). In contrast, Eldridge *et al.* (1977) reported normal concentrations of noradrenaline in the plasma of TS patients, and postmortem studies revealed no difference in noradrenaline in the cerebral cortex (Singer *et al.*, 1990) or basal ganglia (unpublished data refer to: Singer and Walkup, 1991).

The association between cholinergic and dopaminergic systems in the brain (Di Chiara *et al.*, 1994), justifies consideration of an involvement of cholinergic mechanisms in the pathophysiology of TS, but the variable clinical response of TS patients to agents affecting this system has provided conflicting results. Intravenous injection of physostigmine improved tic symptoms in some patients (Stahl and Berger, 1981), although an exacerbation of motor symptoms has also been reported after its use (Tanner *et al.*, 1982). Investigations into acetylcholine metabolism have found no difference in CSF acetylcholinesterase or butyrylcholinesterase activity between TS patients and controls (Singer *et al.*, 1984). Similarly, evidence for an abnormality in cholinergic mechanisms has not emerged from postmortem brain studies, since the activity of choline acetyltransferase, the synthesizing enzyme for acetylcholine, and muscarinic receptor binding in TS subjects is unaltered compared with controls (Singer *et al.*, 1990). On the other hand, reports of non-smoking TS patients inadequately controlled by neuroleptics, who exhibited an amelioration of tics after application of transdermal nicotine patches, suggests a possible role for the nicotinic cholinergic pathway in the aetiology of TS (Silver and Sanberg, 1993; Dursun *et al.*, 1994b; Silver *et al.*, 1996).

Speculation of a GABA influence in TS arose from the efficacy of the benzodiazepine clonazepam to suppress tics in some patients (Gonce and Barbeau, 1977; Goetz, 1992). Apart from slight increases in GABA concentrations in the medial and lateral globus pallidus from postmortem TS brains, Anderson *et al.* (1992) found no evidence of an abnormality. Likewise, the activity of glutamate decarboxylase, which catalyses the conversion of L-glutamic acid to GABA and serves as a presynaptic marker of GABAergic interneurones in the cerebral cortex, is normal in postmortem cortex in TS (Singer *et al.*, 1990), as are levels of GABA in whole blood and CSF (Van Woert *et al.*, 1982).

A deficit of glutamate in the medial globus pallidus, lateral globus pallidus and substantia nigra in postmortem brain tissue (Anderson *et al.*, 1992) was suggestive of a possible glutamate dysfunction in TS, although no further evidence has been reported.

The significance of tryptophan (TRP) metabolic pathways (see: The kynurenine pathway and its significance to neuropsychiatric diseases) to TS have been extensively investigated, via human and animal studies, and will be greatly expanded upon in other areas of this thesis. Decreased CSF concentrations of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), has been found in some TS patients (Cohen *et al.*, 1978; Butler *et al.*, 1979), although urinary levels of 5-HT and 5-HIAA between TS children and matched controls showed no difference (Bornstein and Baker, 1990). In another study, Bornstein and Baker (1992a) divided the TS group of children in two subgroups based on whether they had a high or low incidence of OCD symptoms. There were no biochemical differences between the two subgroups, but urinary concentrations of 5-HT and indoleacetic acid (a breakdown product of the TRP metabolite tryptamine) were significantly lower in both of the TS patient subgroups compared with normal controls. This latter result was replicated in an adult study involving TS patients with matched controls (Bornstein and Baker, 1992b). In postmortem studies, Anderson *et al.* (1992) reported a deficit of 5-HT in 11 of 13 brain areas, and reduced TRP and 5-HIAA in 12 of 13 areas compared with controls. Anderson and colleagues suggested that these differences may reflect an alteration of TRP transport or metabolism.

Decreased dynorphin A (1-17) immunoreactivity was found in striatal fibres projecting to the globus pallidus in 4 out of 5 postmortem TS brains (Haber *et al.*, 1992). On the other hand, elevated CSF levels of prodynorphin A (1-8) was demonstrated in a group of TS patients (Leckman *et al.*, 1988), which correlated with severity of OCD symptoms but not tic severity in these subjects. Furthermore, conflicting results has been generated from various case reports of TS patients treated with opioid agonists and antagonists (see: Pharmacological comparisons between TS treatments and animal shaking behaviours).

10. An overview of neopterin - a natural pteridine

The class of compounds known today as pteridines refers to the bicyclic nitrogenous ring system *pyrazino-(2,3-d)-pyrimidine*, which is formally derived from a pyrazine fused with a pyrimidine. Small substituents derived from this parent compound include neopterin and biopterin, otherwise described as 'unconjugated pteridines', whereas derivatives with larger residues, such as folic acid, riboflavin and methanopterin, are termed 'conjugated pteridines'. Pteridines are involved in several important biological processes, for example they function as cofactors in the biosynthesis of neurotransmitters dopamine, noradrenaline and serotonin.

The precursor of natural pteridines is guanosine triphosphate (GTP), which is converted by GTP cyclohydrolase I to 7,8-dihydroneopterin triphosphate, via cleavage of the imidazoline ring of the purine. This key intermediate in pteridine biosynthesis is converted by two further enzymes (6-pyruvoyltetrahydropterin synthase and sepiapterin reductase) to 5,6,7,8-tetrahydrobiopterin, or alternatively, 7,8-dihydroneopterin triphosphate is cleaved by phosphorylases to form 7,8-dihydroneopterin (see: Figure I.1.).

Neopterin occurs in two forms; fully oxidised aromatic neopterin and the reduced species 7,8-dihydroneopterin and 5,6,7,8-tetrahydroneopterin. Problems arise when measuring neopterin in biological samples, due to its unstable nature in the presence of light, and because the reduced form of neopterin is susceptible to oxidative reactions. Consequently, aromatic (or 'native') neopterin is more commonly measured in biochemical studies, rather than 'total' neopterin.

Reports of increased urinary neopterin in patients with malignant tumours and viral infections (Wachter *et al.*, 1979) was central to its recognition as a marker of cellular immune activation, and elevated neopterin levels have since been demonstrated in various other diseases, where a stimulation of the immune system is implicated. The formation and release of neopterin is largely regulated by cytokines, specifically interferon-γ and tumour necrosis factor-α, while macrophages act as the primary cellular source of neopterin. Significantly, interferon-γ induces the activity of GTP cyclohydrolase I, the first enzyme of this catabolic process, and hence the amounts of neopterin and biopterin accumulating in cells depends on the relative activities of GTP cyclohydrolase I and 6-pyruvoyl-tetrahydropterin synthase. In human macrophages, GTP cyclohydrolase I activity exceeds that of 6-pyruvoyl-tetrahydropterin, so that 7,8-dihydroneopterin triphosphate accumulates in macrophages stimulated by interferon-γ. The triphosphate moiety is cleaved by the omnipresent phosphatases, and the resulting molecular species, 7,8-dihydroneopterin and neopterin, can leak from the cells.

These cytokines are also known to cause additional important changes in target cells, for example an increase in major histocompatibility complex (MHC) antigens and induction of indoleamine-2,3-dioxygenase (IDO). As a result, neopterin concentrations from clinical studies are frequently correlated with β_2-microglobulin (a degradation product of MHC-antigen molecules) and the kynurenine pathway metabolites kynurenine (KYN) and quinolinic acid (QUIN).

11. The kynurenine pathway and its significance to neuropsychiatric diseases

The kynurenine pathway is the major route via which dietary TRP is metabolised in mammals (see: Figure I.2.), accounting for approximately 90% of TRP metabolism, the remainder being primarily used in the formation of 5-HT via tryptophan hydroxylase (see: Figure I.3.) (Comings, 1990a). Measurement of KYN provides a gross index of the activity of this catabolic route, which generates several biologically active substances, including 3-hydroxykynurenine (HKY), kynurenic acid (KYNA) and the neurotoxin QUIN (reviewed in: Heyes, 1993; Schwarcz, 1993).

The first and rate-limiting step in the multienzyme kynurenine pathway involves the oxidation of TRP to KYN. In the liver, this reaction is catalysed by tryptophan-2,3-

dioxygenase (TDO), which is induced by its substrate, TRP (Tanaka and Knox, 1959), and also by glucocorticoids, such as cortisol (Knox and Auerbach, 1955) and oestrogen (Rose, 1966). Another substance capable of increasing the turnover of the essential amino acid TRP is the kynurenine pathway end product nicotinic acid, which occurs via an elevation of liver (but not plasma) TRP (Sainio and Sainio, 1990). The extrahepatic enzyme IDO, which catalyses the same oxidative reaction of TRP as its liver counterpart, was later identified in several cells, including macrophages and in brain (Heyes, 1993; Schwarcz, 1993). Although this latter pathway is known to produce QUIN and KYNA, it is uncertain whether it proceeds as far as nicotinamide adenine dinucleotide (NAD) production. IDO is understood to have a wider substrate specificity than TDO and will also metabolise 5-hydroxylated derivatives such as 5-HT (Yamazaki *et al.*, 1985). The activity of the enzyme is not affected by glucocorticoids, but it is strongly induced by cytokines, notably interferon-γ (Wachter *et al.*, 1992). Consequently, levels of circulating KYN are elevated during inflammatory, infectious and autoimmune disorders, which is accompanied by a simultaneous increase in neopterin (Wachter *et al.*, 1992).

Although the liver is viewed as the predominant source of kynurenine pathway metabolites, alterations in some brain kynurenines in certain diseases has prompted a great deal of interest about the production and transport of these substances in the CNS. Studies indicate that cerebral KYN formation and breakdown is regulated, at least to some extent, in localised brain sites. Support for this theory has emanated from the discovery of the existence of several key enzymes of this pathway within brain tissue (Battie and Verity, 1981; Foster *et al.*, 1985), and on the brain accumulation of kynurenine pathway metabolites following systemic administration of precursors (*i.e.* TRP and KYN) (During *et al.*, 1989; Gál and Sherman, 1978; Swartz *et al.*, 1990). It is however, difficult to determine with any precision the proportion of products arising from extracerebral synthesis and uptake (compared with local cerebral formation) after systemic injection of precursors. It is also worth pointing out, that TRP and KYN appear to compete with each other (as well as with other amino acids), for cerebral uptake from the plasma via a carrier mediated uptake system (Fernstrom *et al.*, 1979), so that additional factors such as food intake,

disease and metabolic state, could affect the dynamics of the pathway in the brain and periphery.

Of the many compounds generated by the kynurenine pathway, QUIN and KYNA have excited considerable interest, for their possible roles in the aetiology of several brain diseases, including Huntington's disease, epilepsy and dementia (Schwarcz, 1993). QUIN is a selective agonist at the NMDA class of excitatory amino acid receptors, and this property accounts for its potential as an endogenous neurotoxin. KYNA, on the other hand, is a broad spectrum antagonist of excitatory amino acids, with particularly high affinity for the glycine site of the NMDA receptor complex. In contrast to QUIN, KYNA has demonstrated anticonvulsant and neuroprotective properties in animal models (Foster *et al.*, 1984; Lapin, 1989). It is believed that QUIN and KYNA function as neuromodulators in excitatory neurotransmission, rather than being neurotransmitters, since brain slice studies indicate that neither compound is released following depolarisation, and no mechanism for their removal from the extracellular space has been identified to date (Foster and Schwarcz, 1988; Turski *et al.*, 1988). The highly localised nature of their biosynthetic enzymes in astrocytes has been cited as further evidence for this viewpoint. Exactly in what capacity QUIN and KYNA function has not been clearly defined, but evidence from anatomical studies suggests that local concentrations of QUIN at the synapse may be sufficiently high to influence receptor function (Stone and Perkins, 1984). Moreover, Stone *et al.* (1987) have speculated that if QUIN is continuously released at a low level, it may provide a background of neuronal excitability to the CNS, with adequate removal being achieved by diffusion into the circulation. A regional variation in the sensitivity to QUIN within the brain has also been demonstrated (Perkins and Stone, 1983), which appears to represent the relative proportions of NMDA receptors in the different brain areas. Consequently, since QUIN is a potent excitant and neurotoxin in some brain areas, while in others it appears relatively inactive, in certain disease states it could induce a regionally selective neuronal necrosis, as opposed to a diffuse degeneration of nerve cells (Stone *et al.*, 1987).

The highly polar structures of QUIN and KYNA and lack of a carrier mechanism means that neither substance can normally access the brain (Fukui *et al.*, 1991), and so CNS QUIN and KYNA are most likely synthesized from within the brain.

Consequently, the ability of their precursors (notably KYN) to traverse the blood brain barrier, and the enzymes involved in their formation and breakdown, has attracted particular attention. Hence brain slice work has shown that 3-hydroxyanthranilic acid (HAA), the immediate bioprecursor to QUIN, is preferentially derived from anthranilic acid (AA) rather than HKY (Baran and Schwarcz, 1990), although the source of brain AA is not certain. In addition, quinolinic acid phosphoribosyl transferase (QPRT), the degradative enzyme for QUIN, appears to act as the rate-limiting step in cerebral QUIN metabolism, as opposed to 3-hydroxyanthranilic acid oxygenase (3HAO), which catalyses the formation of QUIN from HAA (Foster and Schwarcz, 1985; Foster *et al.*, 1985). Thus an increased flux of TRP through the kynurenine pathway, could lead to accumulation of QUIN, the consequences of which are unclear. Indeed, an association between immune stimulation within the CNS and an accelerated conversion of TRP to QUIN (via increased IDO activity) has been demonstrated in an animal model of localised inflammatory disease (Heyes *et al.*, 1992b), in which macaques received an intraspinal injection of poliovirus. Increases in IDO activity were accompanied by proportional increases in local tissue QUIN accumulation, as well as a rise in CSF QUIN, KYN and KYNA. Macrophage infiltrates, which have a huge synthetic capacity for QUIN production compared with astrocytes, were identified as the primary source of QUIN. A correlation between CSF QUIN and the macrophage product neopterin in patients with CNS infections, provided further evidence of an activation of macrophages and a proposed link to QUIN accumulations (Heyes *et al.*, 1995). Accordingly, we should not forget the potential significance of KYNA to excitatory neurotransmission. For example, decreases in KYNA function could contribute to overexcitation of receptors, via an imbalance between KYNA and one or more endogenous excitants.

The neuroactive properties of other kynurenine pathway components have been less documented, although HKY, like KYN and QUIN, has been found to induce convulsions when injected into the cerebral ventricles of rodents (Lapin, 1978; Pinelli *et al.*, 1984). Moreover, *in vitro* studies have confirmed the cytotoxicity of HKY (Eastman and Guilarte, 1989; Eastman and Guilarte, 1990), an effect which appears to result from the intracellular accumulation of toxic levels of H_2O_2 (formed via the

oxidation of HKY), rather than a direct action on excitatory amino acid receptors. In addition, endogenously produced HKY concentrations comparable to those which induce these cytotoxic effects, have been produced in the brains of vitamin B_6 deficient neonatal rats (Guilarte and Wagner, 1987). Such increases in HKY arise, because the conversion of KYN to HKY, unlike certain kynurenine pathway steps, is not dependent on the presence of vitamin B_6 (see: Figure 1.2).

The potential importance of the kynurenine pathway to a wide spectrum of disorders means that research into the activity of the pathway and its metabolic products will undoubtedly continue for the forseeable future. Advances in our understanding are likely to be gained by distinguishing the respective roles of blood-derived and brain-derived kynurenines, given that peripheral measurement of these parameters may not be an accurate reflection of what is happening in the CNS.

12. Antineuronal antibodies in neuropsychiatric disorders

In Sydenham's chorea the association between prior streptococcal infection (specifically from group A β-haemolytic streptococci) and the onset of adventitious movements has been well documented (Taranta and Stollerman, 1956). It has been hypothesised, that in this variant of rheumatic fever, antineuronal antibodies arise in response to the streptococcal infection, which cross-react with cells within the CNS, notably basal ganglia tissues (Husby *et al.* 1976), and this immunological reaction is manifested as a movement disorder. More recently, evidence has been presented to suggest that infection with Group A β-haemolytic streptococci could be responsible for the sudden onset or episodic worsening of OCD and/or tic disorders (including TS), via an autoimmune process analagous to Sydenham's chorea (Swedo *et al.*, 1994). In these studies, children with recent onset of movement disorders (tics, TS and choreiform movements) exhibited an increased presence of antineuronal antibodies and a greater incidence of one or more elevated antistreptococcal titres in their serum than children without any motor abnormalities (Kiessling *et al.*, 1993; Swedo *et al.*, 1994). As a result, it was construed that both antineuronal antibody and antistreptococcal antibody status were related to the presence of movement disorders in these patient groups (Kiessling *et al.*, 1993). Further support for this theory was derived from individual case reports showing concomitant appearance of movement

disorders and elevated antistreptolysin O (ASO) titres, in patients diagnosed as having group A β-haemolytic streptococcal pharyngitis (Kiessling *et al.*, 1993). Swedo *et al.* (1994), cited a correlation between symptom exacerbation and increased antibody titres in two small series of patients with TS (who were assessed for more than a year), as a possible explanation for the typical waxing and waning nature of the TS symptoms.

There is also evidence that immunomodulatory treatments may improve symptoms in some patients diagnosed with severe OCD, TS or tic disorders, resulting from a postulated autoimmune aetiology (Allen *et al.*, 1995; Tucker *et al.*, 1996). In one case presented by Tucker *et al.* (1996), a 12 year-old girl diagnosed with OCD and chronic tic disorder was tested positive for group A β-haemolytic streptococci. After undergoing plasmaphoresis and prophylactic penicillin treatment, the patient showed a modest improvement in her tic and obsessive-compulsive symptoms, which appeared to worsen after the penicillin was discontinued.

Although neurological literature has clearly differentiated between tics and chorea, some researchers have proposd that an association between these conditions should not be dismissed entirely (Swedo *et al.*, 1994). Swedo *et al.* (1994) conjectured that specific manifestations of antineuronal antibody-mediated CNS dysfunction might depend on the genetic makeup of the individual, as well as the nature and developmental timing of the immune response, but emanate from a common environmetal trigger (streptococci). For example, the incidence of Sydenham's chorea in girls is three times higher than in boys, whereas TS is more prevalent in boys by a comparable ratio, thereby suggesting that gender differences could play a significant role in symptom expression in both of these neuropsychiatric disorders.

Nevertheless, Swedo and colleagues (1994) acknowledge that the hypothesis of antineuronal antibody-mediated neuropsychiatric dysfunction may only explain a subgroup of children with OCD, tics or TS, since not all children have an episodic course, report an abrupt onset of their symptoms, or have increased antibodies. Further research will be necessary to clarify the importance of an individuals genetic make-up in determining their susceptibilty to these conditions, and the precise mechanism by which such disparate symptoms are expressed in these vulnerable individuals.

13. Animal models of TS

Possibly the most influential factor in helping to define the biochemical basis of any neuropsychiatric disorder, as well as offering potential new avenues for treatment (for which TS is no exception), is the establishment of a suitable animal model for the condition. In order to determine the validity of any such model, a number of criteria must be considered. These comprise: construct validity (a sound theoretical rationale), face validity (distinct similarities between the disorder and the model including pharmacology) and predictive validity (predominantly a prediction of drug actions in the clinic based on their effects in the model), all of which must be satisfied before accepting a model as representing a human disease state (Abramson and Segilman, 1977; McKinney and Bunney, 1969).

Predictive validation of any proposed models of TS has been minimal to date, and assessment of construct validity is somewhat hindered by our poor understanding of the primary neurochemical origin of this disorder. Nevertheless, in light of the success of neuroleptics in treating TS symptoms, many research groups have sought to induce a hyperdopaminergic state for potential models, using agents such as L-dihydroxyphenylalanine (L-DOPA) (Knott and Hutson, 1982) and 6-hydroxydopamine (Friedhoff, 1982; Shaywitz *et al.*, 1982) to modulate dopaminergic activity. However, these models failed to produce tic movements analgous to human tics.

Shaking behaviours as a model of human tics and their pharmacological origin:

Having studied animal behaviours which might resemble the symptoms observed in human TS sufferers, Handley and Dursun (1992) proposed that head-shakes (HS) and 'wet-dog-shakes' (WDS) were highly congruent with human tics. These HS (alternatively known as head-twitches) and WDS behaviours belong to the grooming repertoire, and either or both occur spontaneously in all furred and feathered species (Wei, 1981). Whether spontaneous or induced, the HS is described as a single rapid rotation of the head upon the neck, thereby corresponding to the human head-shake or 'hair-out-of-the-eyes' tic (Handley and Dursun, 1992). The WDS is a single or multiple rotation of the head, shoulders and upper trunk reminiscent of the

movements of a dog emerging from water (Bedard and Pycock, 1977; Wei, 1981), bearing a close likeness to shoulder shrugs and trunk/shoulder rotation tics in humans (Handley and Dursun, 1992). Moreover, it has been suggested that the WDS may represent a more fully expressed form of the HS response (Wilkinson and Dourish, 1991).

Shaking behaviours in rodents have been reported following treatment with several chemically and pharmacologically diverse substances, having potential activity at 5-HT, GABA, acetylcholine, noradrenaline, glutamate and opioid receptors (reviewed by: Handley and Singh, 1986a). This suggests a widespread neurotransmitter modulation of the behaviour. Furthermore, opioid withdrawal, administration of thyrotropin-releasing hormone (TRH) and physical procedures (such as tactile stimulation of the pinna) have proved capable of eliciting shaking behaviours in rodents (Handley and Singh, 1986a).

The characteristic head-twitch was first described in mice (Corne *et al.*, 1963) and later observed in rats (Matthews and Smith, 1980) following administration of the 5-HT precursor 5-hydroxytryptophan (5-HTP), via a central action at 5-HT receptors (Nakamura and Fukushima, 1978; Matthews and Smith, 1980). In addition to 5-HTP, much of the work carried out on these shaking phenomena has employed selective 5-HT agonists, for example 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI).

Strong evidence for a serotonergic link in the mechanism of action, was provided by reports that shaking induced by a wide range of substances was in many cases prevented by 5-HT antagonists (reviewed by: Handley and Singh, 1986a). Handley *et al.* (1986) suggested that these movements were mediated predominantly via 5-HT_{2A} receptors, because ritanserin (a specific 5-$HT_{2A/2C}$ receptor antagonist) reduced tics induced by morphine and clonidine withdrawal in mice. This theory was supported by Kennett and Curzon (1991), after finding a significant correlation between the ID50 values of ten antagonists against 5-HTP (plus carbidopa)-induced HS and their affinities for 5-HT_{2A} receptors. Similarly, Lucki *et al.* (1984) found that the ability of 5-HT antagonists metergoline, ketanserin, pipamperone and methysergide to block quipazine-induced head-twitches in rats was closely correlated with their reported affinities for the 5-HT_{2A} receptor.

Behavioural studies suggest a functional interaction between the 5-HT_{1A} receptor and the 5-HT_{2A} receptor. Neither the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) or the 5-HT_1 agonist RU 24949 produced head twitches in mice (Goodwin and Green, 1985), but 8-OH-DPAT reduced 5-HTP-induced HS (Goodwin and Green, 1985). In addition, HS induced by the 5-$HT_{2A/2C}$ agonist DOI were dose dependently inhibited by 5-HT_{1A} agonists buspirone, gepirone, ipsapirone, (+)-flesinoxan, and 8-OH-DPAT (Dursun and Handley, 1993; Schreiber *et al.*, 1995). Further evidence ensued from the findings that the latter effects of 8-OH-DPAT were blocked by the 5-HT_{1A} receptor antagonist (−)-tertatolol in a dose related manner (Schreiber *et al.*, 1995).

Because *p*-chlorophenylalanine (pCPA), a 5-HT depleting agent, did not affect DOI-induced HS, but prevented the inhibitory actions of 8-OH-DPAT and buspirone (Dursun and Handley, 1993), the authors proposed that the 5-HT_{1A} receptors involved in the antagonism of DOI-HS may be located presynaptically. This view was challenged by Schreiber *et al.* (1995), who reported that pCPA failed to attenuate the inhibitory effects of ipsapirone on DOI-induced HS, and therefore postulated that the post-synaptic 5-HT_{1A} receptors predominantly mediate this 5-HT_{2A}-induced response.

Tic-like movements induced by TRH:

Thyrotropin releasing hormone (TRH) and its analogues induce various tic-like movements in rodents (Dursun and Handley, 1991; Fone *et al.*, 1989). In rats, intrathecal injection of the TRH analogue CG 3509 produced WDS and forepaw-licking behaviours, which were attenuated by pretreatment with ritanserin (Fone *et al.*, 1989). Intracerebroventricular injection of TRH-amide to mice caused rapid blinking, straub-tail, tail tremor, forepaw tremor, head shaking and scratching behaviours (Dursun and Handley, 1991), all of which were significantly antagonised by ritanserin. Haloperidol caused a significant reduction in straub-tail, tail tremor, scratching and HS only (Dursun and Handley, 1991). Particular interest in the blinking feature relates to the observed bursting nature of this behaviour in TS patients (Dursun, Rickards and Handley, unpublished observation), and results

indicating that the mean spontaneous blinking rate in a group of unmedicated TS patients was almost three times higher than for controls (Bonnet, 1982).

Rapid phasic movements coincident with vocalisation:

One obvious shortcoming in the HS and WDS models for TS is the lack of a vocal component, bearing in mind that vocal tics are an essential symptom for the diagnosis of TS (A.P.A., 1995). Dursun and Handley (1994) reported that administration of RX336-M to mice, induced a complex of simultaneous rapid movements of body parts (including whole-body jerk and/or a head-shake), which were coincident with single sqeak vocalisations. The frequency of the complex was dose dependent, and was reduced by haloperidol, as well as the 5-$HT_{2A/2C}$ antagonists ritanserin and ICI 169, 369.

14. Pharmacological comparisons between agents used for treating TS and those which modify animal shaking behaviours

Many of the agents considered as potential TS treatments have been studied for their effects on tic-like movements in animals. A major difference in terms of the methods used for humans and animals may arise, however, because the efficacy of a drug in TS patients is frequently monitored via its effect on a wide range of symptoms (including tics), whereas in animal models the frequency of a single shaking behaviour is quantified.

Neuroleptics:

Haloperidol is predominantly a dopamine receptor antagonist (selectively acting at D_2 receptors), but possesses some adrenergic α_1-receptor blocking properties. It is widely used in the treatment of TS, but discontinuation of therapy by patients can often arise due to its unacceptable adverse effects, which include cognitive impairment, sedation, loss of motivation, weight gain, movement abnormalities (such as akathisia, akinesia and acute dystonia) and depression (Shapiro and Shapiro, 1981; Shapiro and Shapiro, 1982). Haloperidol has also been implicated in the precipitation of tardive dyskinesia (Riddle *et al.*, 1987). Pimozide, which binds preferentially to dopamine D_2 receptors (but with little effect at adrenergic α_1-receptors), is

sometimes used as an alternative to haloperidol. Although less sedating than haloperidol and with a similar efficacy, pimozide is often reserved for patients not responding to haloperidol or who are unable to tolerate its side-effects. Pimozide can also have severe side effects at higher doses and/or with prolonged use, and electrocardiogram changes have been reported in some patients receiving pimozide (Shapiro *et al.*, 1989; Cohen *et al.*, 1992). The misgivings associated with haloperidol and pimozide could explain the interest in the use of the selective D_2 antagonist sulpiride in TS treatment, although its efficacy has not been fully evaluated (Robertson *et al.*, 1990). Reductions in WDS and spontaneous and 5-HT-related HS by neuroleptics have been demonstrated (Matthews and Smith, 1980; Arnt *et al.*, 1984; Handley and Dursun, 1992), but their potency in attenuating these tic-like behaviours is correlated to their affinity for 5-HT_{2A} and α_1-receptor sites (Dursun and Handley, 1992).

Clonidine:

Clonidine, an α_2-adrenergic receptor agonist, has provided a useful alternative to neuroleptics, although figures suggest that the proportion of patients gaining benefit from the drug are lower than for haloperidol, typically ranging from 20-40% (Singer *et al.*, 1985-86; Leckman *et al.*, 1985; Leckman *et al.*, 1991; Cohen *et al.*, 1992). Clonidine exhibits a more favourable (and generally milder) spectrum of side effects than neuroleptics, the most commonly reported being sedation, dry mouth, faintness, dizziness and irritability (Leckman *et al.*, 1991). Studies indicate that clonidine and other α_2-agonists reduce spontaneous and drug-induced HS in mice (Bednarczyk and Vetulani, 1978; Handley and Brown, 1982; Heal *et al.*, 1986; Dursun and Handley, 1992), and conversely α_2-receptor antagonists potentiate HS frequency (Handley and Brown, 1982; Heal *et al.*, 1986).

5-HT agonists and antagonists:

In view of the apparent importance of serotonergic mechanisms to shaking behaviours in rodents, there is a paucity of investigative literature documenting the effects of such agents in human clinical studies for TS, and in particular drugs having selectivity for 5-HT receptor subtypes. Methysergide, a non-selective antagonist,

produced an initial improvement of tic symptoms in two cases of TS, but the benefits proved to be temporary (Shapiro and Shapiro, 1981). Chandler *et al.* (1989) reported two cases of ADHD children, one with pre-existing tics that were aggravated by stimulants and the second with stimulant-induced tics. A combined methylphenidate/*L*-TRP treatment resulted in a sustained improvement of ADHD symptoms, as well as abolishing motor and phonic tics. In contrast, Shapiro and Shapiro (1981) found that the 5-HT precursor, *L*-TRP, had no effects on tics in two patients. More recently, Dursun *et al.* (1995) reported an improvement in tic symptoms following buspirone treatment in one TS patient, who had not responded to or tolerated antipsychotic medications. Although this result compares favourably with behavioural studies, which found a reduction of DOI-induced HS frequency in mice pretreated with buspirone (Dursun and Handley, 1993; Schreiber *et al.*, 1995), replication of this effect is obviously warranted.

Uptake inhibitors:

Tricyclic antidepressants, which predominantly block the noradrenaline uptake mechanism, have proved ineffective in TS treatment (Messiha, 1988). Selective serotonin reuptake inhibitors have been investigated on the basis of their reported efficacy in the treatment of OCD, (Goodman *et al.*, 1990, Riddle *et al.*, 1990 McDougle *et al.*, 1993), which frequently coexists in TS patients (see: Associated features). However, there is little evidence that serotonin reuptake inhibitors significantly improve TS symptoms (Riddle *et al.*, 1990; Scahill *et al.*, 1997), and in some cases a worsening of tics has been reported following their use (Delgado *et al.*, 1990; Gatto *et al.*, 1994). In behavioural studies, twice daily administration of citalopram to mice for a period of 21 days initially potentiated spontaneous HS frequency, which was later followed by a reduction in HS (from day 4), before returning to basal levels in the latter days of the study (Handley and Dursun, 1992).

Nicotine:

Subsequent to finding a significant potentiation of haloperidol-induced catalepsy in rats by systemic nicotine injection (Sanberg *et al.*, 1989), Sanberg and colleagues reported that concurrent administration of 2mg nicotine gum reduced tics in 10 TS

patients (age range 7 to 17 years old) being treated with haloperidol, although nicotine gum alone had no effect. The therapeutic effects of nicotine gum were of short duration, and led to a high incidence of side effects, including stomach pains, nausea, decreased appetite and complaints about a bitter taste. In subsequent studies, improved symptomatology was demonstrated by non-smoking TS patients, following application of a transdermal nicotine patch either alone or in conjunction with haloperidol (Silver and Sanberg, 1993; Dursun *et al.*, 1994b; Silver *et al.*, 1996). The main adverse effects associated with the nicotine patches were transient itching at the site of application, nausea, headache and sedation (Silver *et al.*, 1996). Moreover, there was considerable variation in the extent and speed of tic symptom improvement, and in the period of therapeutic effect gained by patients following nicotine patch removal (Dursun *et al.*, 1994b; Silver *et al.*, 1996). In addition, animal studies have shown that WDS in rats induced by systemic administration of the glutamate analogue kainic acid are significantly reduced by subcutaneous nicotine pretreatment (Shytle, *et al.*, 1995). The mechanism of action of nicotine in these human and animal investigations, however, remains unclear.

Kynurenine:

Elevated plasma KYN concentrations have been reported in TS patients (Dursun *et al.*, 1994a). Studies carried out by Handley and Miskin (1977) showed that kynurenine pathway metabolites KYN, HKY and xanthurenic acid (XANA) had no effect on spontaneous HS in mice. However, 5-HT and 5-HTP-induced HS were potentiated by low doses of KYN and HKY, whilst at higher doses of KYN and HKY both responses were antagonised (Handley and Miskin, 1977). More recently, an increase in DOI-induced HS frequency in mice was found after low dose KYN pretreatment (McCreary and Handley, 1995). The mechanism of this KYN response has not been elucidated, since ligand binding studies indicate that none of the kynurenine pathway metabolites bind to 5-HT_{2A} or 5-HT_{1A} receptors (Kariyawasam *et al.*, 1997).

Other agents:

A variety of other agents have been investigated for their potential benefits in tic conditions, but with limited success. Messiha (1988) reported beneficial effects of lithium, but only after prolonged treatment, whereas other research groups have found little therapeutic benefit (Shapiro and Shapiro, 1981; Borison *et al.*, 1982). Interestingly, very high doses of lithium (given acutely) can induce HS in rodents (Handley and Singh, 1986a).

The ability of morphine withdrawal to induce shaking behaviour in mice is well known (Handley and Singh, 1986a; Handley *et al.*, 1986). However, case reports suggesting opioid dysfunction in TS have provided conflicting results. Symptomatic improvement has been observed with opioid antagonists naloxone and naltrexone (Sandyk, 1985; Kurlan *et al.*, 1991; Chappell *et al.*, 1992), and with the opioid agonist methadone (Meuldijk and Colon, 1992). Opposite effects were demonstrated by the selective κ-opiate agonist spiradoline at different doses (Chappell *et al.*, 1993). In addition, motor and phonic tic exacerbation was found in one patient after sudden withdrawal of chronic opiate therapy (Lichter *et al.*, 1988).

Benzodiazepines have not shown significant therapeutic benefit in TS (Connell *et al.*, 1967; Shapiro and Shapiro, 1981), and in rodents diazepam potentiates HS induced by 5-HTP, quipazine and 5-MeODMT (Handley and Singh, 1985; Handley and Singh, 1986b; Moser, 1993).

15. Aims of the project

The initial aim of this project was to investigate, via an initial pilot study, the effects of diet and diurnal factors on kynurenine pathway parameters, and to replicate the previous findings from Dr. Handley's research group of increased plasma KYN concentrations in TS (Dursun *et al.*, 1994a). Subsequently, by means of a large cross-sectional study, it was our intention to explore the mechanism via which KYN might be altered in TS, by measurement of certain kynurenine pathway substances in the plasma of TS patients and matched controls, as well as plasma neopterin and cortisol (to help monitor the activities of IDO and TDO respectively). The third part of these human studies involved an assessment of TS symptoms and kynurenine pathway parameters in a group of TS patients, at monthly intervals over a 5 month period, to

investigate the possible association between symptom severity and certain biochemical variables. Finally, we hoped the animal HS model could be exploited to further our understanding of the underlying pathophysiology of tic behaviours. The results from both the human and animal work might enable us to identify new avenues for research, and ultimately lead to the introduction of more acceptable and efficacious treatments.

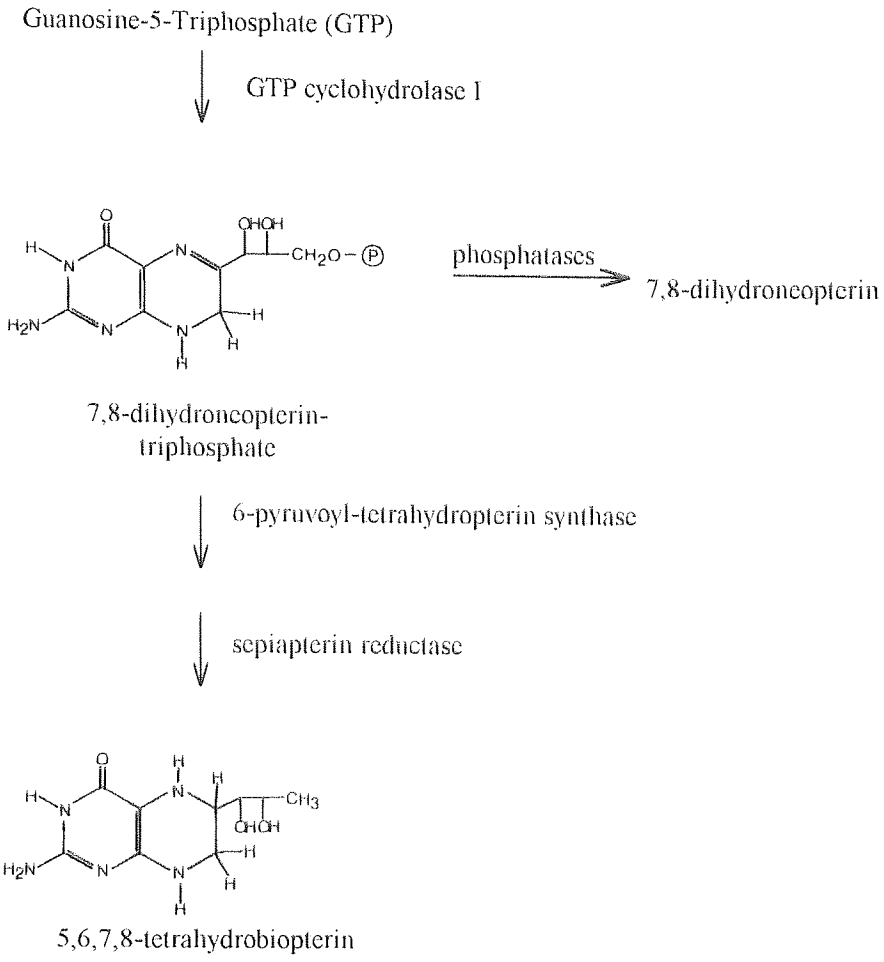

Figure I.1. Simplified scheme of pteridine biosynthesis from guanosine triphosphate (Wachter *et al.*, 1992).

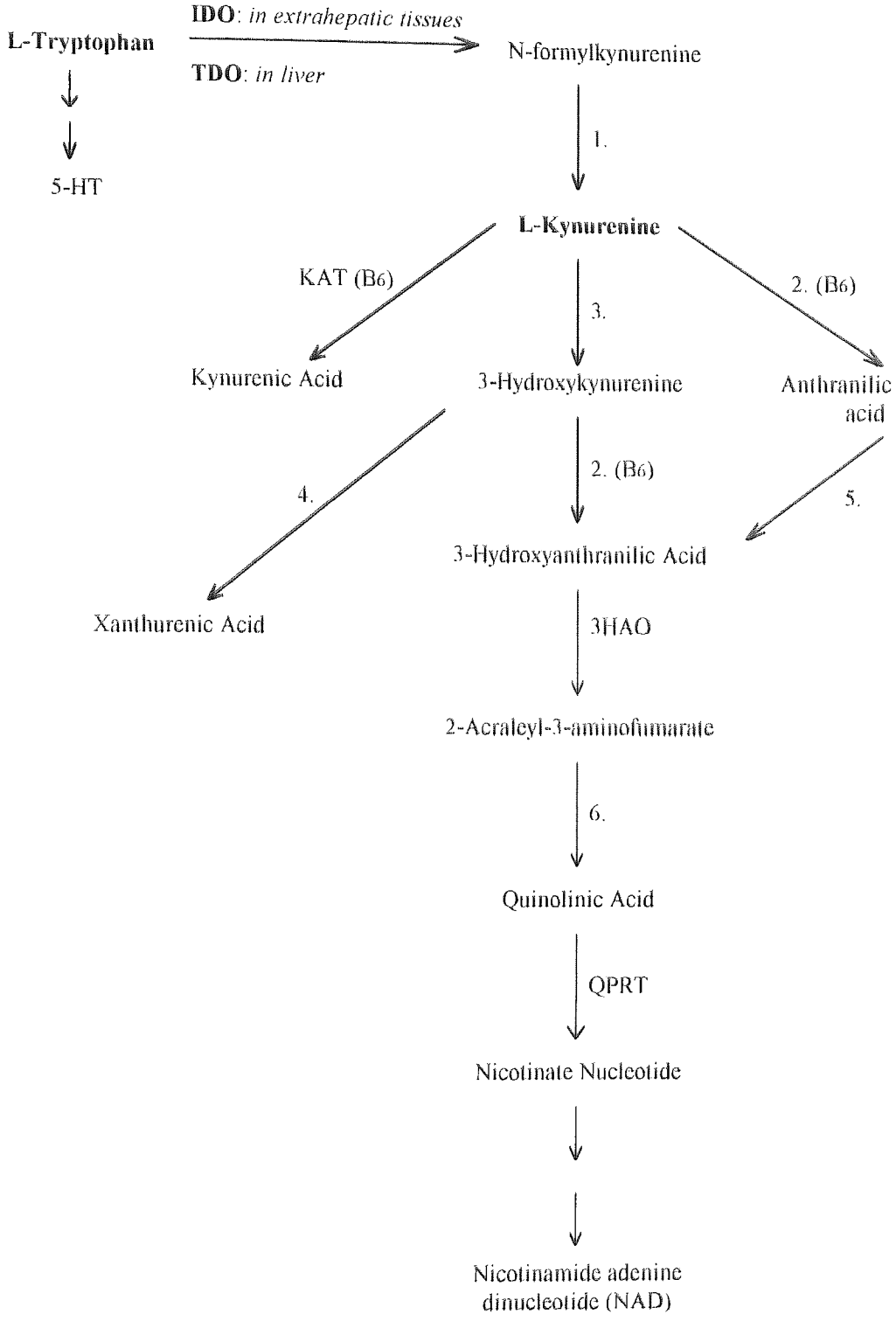

Figure I.2. General overview of Tryptophan metabolism via the Kynurenine pathway.

Key:
IDO = Indoleamine-2,3-dioxygenase; TDO = Tryptophan-2,3-dioxygenase;
KAT= Kynurenine aminotransferase; 3HAO = 3-Hydroxyanthranilic acid oxygenase;
QPRT = Quinolinic acid phosphoribosyl transferase; B6= vitamin B6 dependent step;
1 = Kynurenine formamidase; 2 = Kynureninase; 3 = Kynurenine hydroxylase;
4 = Transamination; 5 = Non-specific hydroxylation; 6 = Non-enzyme cyclization.

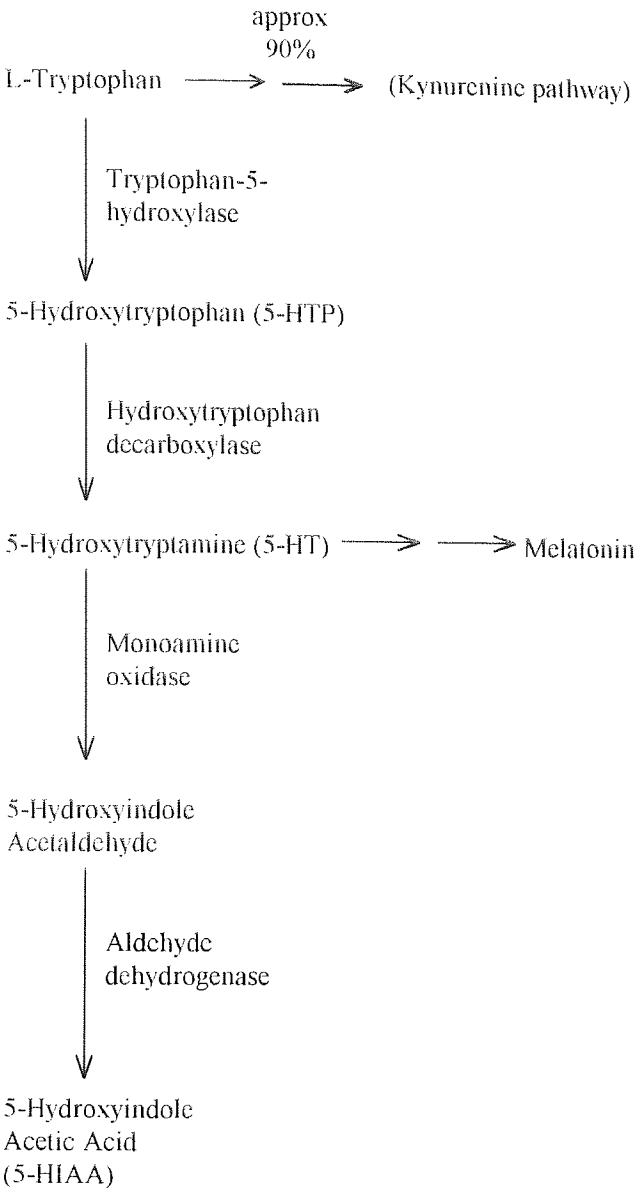

Figure I.3. The main metabolic pathway for the synthesis and metabolism of serotonin.

GENERAL METHODS

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GENERAL METHODS

1. General responsibilities

The recruitment of TS subjects for all investigations within this thesis was carried out by Dr. Hugh Rickards and Dr. Mary M. Robertson. The task of acquiring control volunteers was largely undertook by myself (Colin Gaynor) and Dr. Sheila L. Handley, except for volunteers below the age of 16 years, where Dr Hugh Rickards assumed the responsibility.

Clinical assessments, for which a qualified Physician was required, were performed by Dr. Hugh Rickards and Dr. Mary M. Robertson.

All biochemical assay development, assay procedures and behavioural assessments were performed by myself, unless stated otherwise.

2. Clinical aspects

2.1. Selection criteria for controls

It was necessary that members of control sample groups were physically healthy, free from current infections and with no history of neurological or inflammatory diseases, since some of the biochemical parameters under investigation, are likely to be sensitive to differences in people's immune systems. Additional exclusion criteria stated that controls should have no personal or family history of a tic disorder, obsessive compulsive disorder or related psychopathologies, due to their possible hereditary background. The controls were selected whereby ages and genders were similar to those of the subjects.

The staff and students of Aston University provided the principal source for adult controls in these studies. Volunteers were obtained by means of poster advertisements around Aston University, and assesed for suitability according to the exclusion criteria detailed above. This procedure led to a total of 30 volunteers being enlisted for the main cross-sectional study (see: Chapter 2). The problem of recruiting sufficient controls below the age of 16 years was more complicated. It was not considered ethically justified to venesect healthy children solely for the purpose of this study, and thus our aim was to select groups of children who fulfilled the forementioned criteria, but were already having blood taken for other reasons. This

obstacle was partly overcome by recruiting children who were undergoing cold orthopaedic surgery at Birmingham Childrens Hospital, from which a total of 7 controls were obtained (for main cross-sectional study, see: Chapter 2). For the reasons already discussed, it was necessary to ensure that children who were having surgery for inflammatory conditions (such as rheumatoid arthritis) were excluded. The second group of controls below the age of 16 came from Birmingham Childrens Hospital Medical Day Unit for allergy testing, where it was important that blood was taken from the children prior to challenge with potential allergens. A further 9 samples (for main cross-sectional study) were provided by this approach.

2.2. Selection of TS subjects

All TS subjects were diagnosed according to DSM-IV (A.P.A., 1995). Those patients recruited for the initial pilot study (see: Chapter 1) and the sequential study (see: Chapter 3) were required to attend the Queen Elizabeth's Psychiatric Hospital (Edgbaston, Birmingham) to provide the necessary blood samples. The plasma samples for the main cross-sectional study were given primarily by TS patients at the National Hospital (Queens Square, London) (n=65), although a small number was provided by patients at Queen Elizabeth's Psychiatric Hospital (Birmingham) (n=7). Those involved in the main study also underwent a full examination of motor and vocal tics, echophenomena, coprophenomena, obsessive-compulsive behaviour (OCB), ADHD, self-injurious behaviour and other associated symptoms of TS (as detailed under: Assessment tools).

Both TS subjects and controls were also asked to make known any recent signs of cold symptoms/infections experienced around the period of blood sample collection, which enabled their potential effects on the biochemical variables (particularly neopterin and KYN) to be assessed.

2.3. Assessment tools

The following rating mechanisms were used to diagnose or quantify the nature of various symptoms and associated features in TS subjects involved primarily in the main cross-sectional study (Chapter 2). These assessments were conducted by a

suitably qualified physician (see: General responsibilities), with the exception of those designed for self-assessment.

National Hospital Tourette Questionnaire (Robertson and Eapen, 1996):
This highly detailed, observer rated questionnaire measures all the main and accessory symptoms of TS, including age and mode of onset of the disorder and a cumulative inventory of motor and vocal tic types. Family history, medical and treatment records and the presence, type and severity of comorbid behaviours are also documented. TS subjects in the main cross-sectional study (Chapter 2) were defined as having obsessive-compulsive behaviour (OCB) if they satisfied any of the 'obsessional' or 'compulsive' criteria detailed in the National Hospital Tourette Questionnaire.

Yale Global Tourette Severity Score (YGTSS) (Leckman et al., 1989):
This is another observer rated instrument which assesses TS severity based upon tic frequency and severity, complexity, diversity of tic types, interference with activities and overall effect on daily life. The inventory gives a final score out of 55, with motor and vocal tics being evaluated separately.

Beck depression inventory (BDI) (Beck et al., 1961):
The BDI is a patient rated inventory which measures depressive symptoms, and was used in the study to provide a measure of depressive symptomatology, rather than for assessing 'caseness' of depression. This test was carried out by subjects and controls.

Spielberger state-trait anxiety index (STAI) (Spielberger et al., 1970):
This self-assessed questionnaire was used to indicate the state of anxiety in controls and subjects (at time of blood sample collection). The decision to include this assessment was made on the basis of studies, which indicated a correlation between anxiety and plasma KYN concentrations (Orlikov *et al.*, 1994). Thus increased levels of anxiety could give rise to elevated KYN, possibly via stress-induced increases in plasma cortisol leading to enhanced TDO activity.

Leyton obsessional inventory (LOI) (Cooper, 1970):

Subscores for 'symptoms' and 'traits' as well as a total score for obsessions and compulsions are provided by this extensive self-rated questionnaire, which was employed for subjects and controls.

Measures of ADHD:

The diagnostic criteria used were DSMIII-R (A.P.A., 1987), which was later changed at the London Clinic to DSMIV (A.P.A., 1995). The test was used to generate a score in affected TS subjects based on symptoms of inattention, hyperactivity and impulsivity.

2.4. Collection and storage of plasma samples

Venous blood samples were taken aseptically and placed in lithium-heparinised tubes. Whole blood was centrifuged at 3500 x g for 10mins. The plasma supernatant was transferred into labelled Eppendorfs in volumes of 0.50ml and stored at -70°C, until being assayed. The samples were all coded and the assays performed blindly with respect to clinical diagnosis.

3. Biochemical procedures

3.1. HPLC apparatus

The HPLC apparatus was a spectrasystem model consisting of a P2000 gradient pump (Spectra-Physics Analytical, Fremont, California, U.S.A.) with an SCM400 vacuum degasser (Spectra-Physics Analytical) connected to an AS3000 autosampler (with a tray temperature control option) equipped with a 100µl variable pushloop injection system (Spectra-Physics Analytical). The detectors consisted of a variable wavelength ultraviolet (UV) absorbance detector (Thermo Separation Products, Fremont, California, U.S.A.), an FL2000 fluorescence detector (Thermo Separation Products) and an ANTEC DECADE electrochemical detector (ECD) (Leiden, Netherlands). The detectors were linked via an electronic integrator (Spectra-Physics, Fremont, California, U.S.A.) to a PC1000 computer using OS/2 software (version

1.30.2: International Business Machines Corporation, *i.e.* IBM), which was programmed for calculating peak areas and retention times.

All chromatographic separations were performed using an Ultra Techsphere 5-ODS column (250mm x 4.6mm I.D., particle size 5µm). The analytical column was fitted with 1cm x 3.0mm I.D. guard column with 5µm C_{18} packing (Techsphere). Both the analytical and guard columns were obtained from HPLC Technology.

All mobile phases were routinely degassed by vacuum filtration through a filter of 0.2µm pore size.

3.2 Initial HPLC background work

The early HPLC work was carried out to try to establish methods capable of measuring each of the kynurenine pathway substances of interest. Initially we sought to replicate the method described by Bizarri *et al.* (1990) whereby seven of these pathway substances were quantified in urine samples using UV detection (at wavelength 254nm). Although we were successful in achieving satisfactory resolution of the standards, UV detection lacked the sensitivity required for measuring KYNA, HKY, HAA, AA and XANA in human plasma. KYNA, HKY and HAA were eventually measured in plasma according to the methods detailed below, but all attempts to quantify AA and XANA proved unsuccessful, due to insufficient sensitivity. Problems were also encountered in trying to quantify KYN in plasma at this wavelength setting, predominantly due to a huge all-consuming solvent front, as well as numerous interfering peaks. The KYN absorption spectrum (see: Fig. 1) subsequently indicated another absorption peak for the compound at 360nm. The problem of interfering peaks was thus overcome (because fewer compounds absorb at this wavelength), and since the necessary sensitivity was also achieved, measurement of KYN was carried out at 360nm (see method below: section 3.4). Fluorescence detection was employed to quantify plasma TRP levels (in spite of the wavelength switching option on the HPLC system), since it reduced the likelihood of interfering peaks. A luminescence spectrometer (Perkin Elmer Ltd, Buckinghamshire, England) was used to determine the optimum wavelength settings for TRP measurement (excitation wavelength, 304nm; emission wavelength of 400nm).

We were unable to detect QUIN using any HPLC techniques, even using QUIN standard solutions prepared at extremely high concentrations. However, most assay procedures for QUIN determination referred to in the literature (for example Heyes *et al.*, 1995) used gas chromatography-mass spectrometry procedures to measure QUIN, which were unviable due to lack of equipment or the necessary expertise.

For each of the HPLC procedures described below, concentrations of kynurenine pathway substances were corrected for on the basis of their recovery from plasma extracts (all values quoted where possible). To account for changes in environmental conditions on different days of assay runs (within a particular experiment), individual aliquots of plasma, from a pooled plasma sample, were included during each particular batch of HPLC assays, unless a suitable internal standard was available. Wide deviations from the values expected for peak areas or recovery values resulted in a sample run being repeated.

3.3. Treatment of plasma samples for HPLC analysis

The procedure used for the treatment of plasma samples, prior to measurement of the relevant kynurenine pathway substances was essentially the same for each assay method. After allowing the plasma samples to thaw, ice cold perchloric acid was added to the plasma aliquot to give a concentration of 5% v/v perchloric acid (within a standard volume appropriate for that assay). The samples were left on ice for 10 minutes to aid deproteinisation, which was followed by centrifugation at 14,000 x g (temperature 4°C) for 15 minutes, to enable separation of the precipitated protein. The resulting supernatant was injected directly onto the HPLC under the conditions described below.

The tray cooler option on the autosampler of the HPLC system enabled the samples to be maintained at a temperature of 4°C, which was of considerable importance, given the poor stability of these compounds.

3.4. HPLC conditions for measurements of KYN and TRP in plasma

The mobile phase consisted of a 10mM sodium acetate buffer containing 3% acetonitrile, pH 4.75 (adjusted with glacial acetic acid). The flow-rate was 1.0 ml/min

and the volume of injected plasma extract was 100μL. KYN was detected by UV absorption with the detector wavelength set at 360nm. TRP was measured using a fluorescence detector (excitation wavelength, 304nm; emission wavelength, 400nm) in series with the UV detector. The total time for each plasma extract run was 36 mins. Figures II and III illustrate typical chromatograms for plasma extracts under the conditions described, using the UV and fluorescence detectors respectively.

There was a linear relationship between the amounts of KYN and TRP standards injected and the detector responses. The lowest detectable amount of KYN standard was 12.5pmol (see: Figure IV) and the corresponding value for TRP was 20pmole (see Figure V).

Verification of their peaks in plasma extracts was achieved by addition of known amounts of KYN and TRP standards to aliquots of native plasma, which resulted in increases in peak areas directly related to the amount of added standard (see: Figures VI and VII).

The elution time was 7.1 mins for KYN and 12.7 mins for TRP. The recovery levels of KYN and TRP respectively was 112.0 ± 1.9 % and 75.0 ± 2.1 % (Mean ± SEM; n=12).

3.5. HPLC-fluorescence measurements of KYNA in plasma

The following method for determination of KYNA concentrations in plasma was based on the procedures described by Shibita (1988), Heyes and Quearry (1990) and Swartz *et al.* (1990). The adapted schedules utilised a mobile phase of 8% acetonitrile in 50 mM sodium acetate, pH 6.0 (adjusted with glacial acetic acid) and pumped at a flow rate of 1.0 ml/min. KYNA separated under these conditions was treated with 0.5 M zinc acetate (used as a post-column reagent), delivered at a flow rate of 1.0 ml/min by a second pump (LDC/Milton Roy constametric III metering pump). The injection volume was 100 μl. The fluorescence detector was set at an excitation wavelength of 254 nm and an emission wavelength of 398 nm. The retention time of KYNA under these conditions was 6.2 mins and the total HPLC run time was 14 mins, as illustrated by the chromatogram in Figure VIII.

Zinc acetate was used as a post column reagent to increase the fluorescence of KYNA, since reports indicate that Zn^{2+} markedly enhances the fluorescent yield of KYNA by up to 50-fold (Shibita, 1988; Swartz *et al.*, 1990).

The calibration curve for KYNA proved linear in the range 0.31 to 10 pmol per injection (see: Figure IX), with a detection limit of 0.31pmol. The recovery of KYNA from human plasma was 67.1 ± 1.4% (mean ± SEM; n=12).

To investigate whether the KYNA signal in the plasma samples is influenced by constituents of plasma, known amounts of KYNA standard were added to aliquots of pooled plasma. The resultant increases in the KYNA detector response (*i.e.* peak area) was directly proportional to the amount of added KYNA standard (refer to: Figure X).

3.6. HPLC-ECD measurements of HKY and HAA in plasma

Isocratic reversed-phase separation of HKY and HAA in an extract of human plasma (injection volume, 50μL) was achieved using a mobile phase consisting of 0.1M potassium dihydrogen orthophosphate buffer (adjusted to pH 3.4 with glacial acetic acid) containing 2.5mM octylsulphonic acid, 0.05mM EDTA and 1% acetonitrile flowing at 1ml/min. The ECD was set at a working potential of + 0.60V for quantification of these kynurenine pathway components, and the total time for each extracted plasma HPLC run was 36 min. The method was adapted from Pearson and Reynolds (1991).

Under the described conditions, the retention time for HKY was 19.8 min and for HAA it was 22.1 min (see: Figure XI), as verified by comparison with authentic standard peaks. Calibration plots for HKY and HAA (range 39 to 1250 fmol per injection) exhibited a linear relationship (see: Figures XII and XIII). The limits of detection were 39 fmol for both HKY and HAA.

Recovery from standards added to plasma was 94.7 ± 2.0 % and 98.8 ± 1.6 % (mean ± SEM; n=6) for extracted samples of HKY and HAA respectively. Linear plots for amount of standard added versus response were demonstrated for plasma samples spiked with known amounts of HKY and HAA standards (see: Figures XIV and XV), thereby discounting any influence of other plasma constituents on these peaks of interest.

3.7. Measurement of KYN in plasma (by Joseph & Risby assay, 1975)

The following assay for measurement of KYN was carried out as decribed by Joseph and Risby (1975). The early steps were scaled down to 50% volumes, due to insufficient volumes of plasma being available in the samples of the pilot study (Chapter 1).

2.5ml of plasma was made up to 4.5ml with distilled water and then 0.5ml of perchloric acid/tiron (0.9M perchloric acid containing 50mM tiron) reagent was added. After thorough mixing, the samples were left to stand for 10 minutes, and then centrifuged for 20 minutes at 2500 x g. 1ml of 10M NaOH was added to 3.75ml of the resultant supernatant, and an extraction was performed using 0.5ml of amyl alcohol. A 4ml volume of the aqueous phase was then heated in a boiling water bath for 20 minutes in a closed tube. After cooling and repeating the extraction with 1ml amyl alcohol, 0.85ml of the organic phase was retained and back extracted with 0.6ml 1M HCl. To 0.5ml of the aqueous phase (in a 0.5ml microcuvette) was added 125µl of 3M NaOH, followed by 10µl of sodium nitrite (5g/L). This was proceeded by the addition of 10µl ammonium sulphamate (25g/L) after 5 minutes, followed by 20µl naphthylethylene diamine dihydrochloride (2.5g/L; made up in 95% ethanol) a further 5 minutes later. Thorough mixing was carried out after each step, and 3.5 hours after the final addition of reagent, the absorbance of each sample was read at a wavelength 560nm, via a UV/VIS spectrophotometer P48700 series (Philips Scientific Analytical Division, Cambridge, England). The KYN concentration was calculated by comparison with blank samples and samples containing known amounts of KYN standard.

3.8. Measurement of Neopterin

Determination of plasma neopterin concentrations were performed by HPLC at University of Innsbruck (Austria) courtesy of Professor D. Fuchs. 'Native' (*i.e.* aromatic) neopterin was measured, rather than 'total' neopterin, the reasons for which are detailed in the General Introduction.

3.9. Measurement of Cortisol

Cortisol was measured by a radioimmunoassay procedure known as Coat-A-Count® (available from: Diagnostics Products Corporation, Los Angeles, USA). The Coat-A-Count Cortisol procedure is a solid-phase radioimmunassay, in which ^{125}I-labelled cortisol competes for a fixed time with cortisol in the plasma sample for antibody sites. Because the antibody is immobilised to the wall of a propylene tube, decanting the supernatant serves to terminate the competition and to isolate the antibody-bound fraction of the radiolabelled cortisol. Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the cortisol present in the patient sample.

Assistance was provided by Dr F. Sidey and Dr S. H. Kariyawasam to carry out the cortisol determinations.

3.10. Measurement of anti-streptolysin O in human plasma

RapiTex® ASL diagnostic kit was used for qualitative and semi-quantitative detection of anti-streptolysin O in human plasma. Streptolysin O is an extracellular metabolite of Group A, C and G streptococci, and the test is based on immunochemical reaction between the corresponding antibody and latex particles coated with anti-streptolysin O (ASO). An elevated ASO content leads to visible agglutination of the latex particles. Since this metabolite has been found to be elevated in 80-85% of cases, it thus provides information about recent streptococcal infections. For specific diagnosis of streptococcal infections, the World Health Organisation (WHO) recommends use of ASL and anti-DNase B tests, since the latter moiety is secreted only by Group A streptococci.

The patient sample ASO content was estimated by diluting those plasma samples which proved positive after initial testing for agglutination. The test-kit manufacturers indicate that 200IU/ml is accepted internationally as the upper limit of the "normal range", and that this value is seldom exceeded in persons without clinical symptoms, among whom streptococcal infections were suspected. A two-fold or greater rise in ASO titre is considered significant in all age groups.

These tests were kindly carried out by Dr S. H. Kariyawasam.

3.11. Dissection of brains and collection of blood from mice for biochemical assay

After assessment of spontaneous HS frequency (method described below), blood was obtained from each mouse by cardiac puncture under general anaesthesia, placed in chilled heparinised tubes and centrifuged at 3500 x g for 15 minutes. Immediately after collection of blood, each mouse was killed by cervical dislocation, the whole brains were promptly removed from the skull and the cerebellum dissected out. The wet weight of the dissected brain (minus cerebellum) was quantified with the aid of a torsion balance, then snap frozen in liquid nitrogen before being transferred to a freezer (maintained at -70°C) for storage until being assayed (within 1 month). Meanwhile, the plasma supernatant produced by centrifugation of the blood was transfered to labelled Eppendorfs and stored at -70°C for subsequent measurement of plasma kynurenine pathway substances.

Collection of blood by cardiac puncture was kindly performed by Mr. M. Gamble.

3.12. Measurement of 5-HT and 5-HIAA in mouse brains by HPLC - ECD

To enable measurement of 5-HT and 5-HIAA, the brains were defrosted, individually transferred to a 5ml glass tube, and manually homogenised in 2mL of cold 0.1M perchloric acid containing 0.4mM sodium metabisulphite (antioxidant) and 1.0μM isoprenaline (internal standard) for 2 minutes. Care was taken to ensure that any heat generated by this procedure was kept to a minimum, due to the unstable nature of these compounds. The homogenate was left on ice for 10 minutes (to aid deproteinisation) followed by centrifugation at 14,000 x g (at 0°C) for 10 minutes. The resulting supernatant was injected onto the HPLC (injection volume, 40μL) to allow 5-HT and 5-HIAA levels to be quantified. Excess supernatant was frozen and stored at -70°C for subsequent KYN measurement (under the HPLC conditions previously described for human plasma).

5-HT and 5-HIAA were separated by reversed phase HPLC (column details as before) and measured by ECD with the working electrode set to +0.70V. The peak areas were used to calculate the molar content of 5-HT and 5-HIAA per gramme of brain, and intra-assay variation was corrected for via the internal standard. The

mobile phase consisted of 0.1M sodium dihydrogen orthophosphate, 2.8mM sodium octylsulphonic acid and 0.7mM EDTA, which was adjusted to pH 4.5 using orthophosphoric acid, and then methanol added to a final concentration of 15%. The mobile phase was pumped at a flow rate of 1.2ml/min. This method was adapted from Heal *et al.* (1993).

The retention times of 5-HIAA, ISO and 5-HT were 10.3, 20.3 and 36.8 mins respectively (see Figure: XVI), and the total run time was fixed at 42 mins. Studies confirmed the existence of a linear relationship between peak area and the amount of 5-HT, 5-HIAA and isoprenaline injected onto the HPLC (refer to: Figures XVII, XVIII and XIX). Detection limit for 5-HT was 1.25 pmol per injection, and 2.5pmol for 5-HIAA. The recovery levels for 5-HT and 5-HIAA respectively were 80.8 ± 3.0 % and 67.7 ± 2.2 % (Mean ± SEM; n=4).

4. Behavioural Methods

4.1. Animals, animal husbandry and laboratory conditions

Experiments reported in this thesis were carried out on male mice of MF1 strain (bred at Aston University), which weighed between 20 and 30g (except mice used in chronic studies where final body weight generally ranged from 35 to 40g). Subsequent to weaning, mice were kept in groups of 20-30 (from the same birth cohort) in propylene cages in the animal house at an ambient temperature of 21°C (± 2°C) under normal lighting conditions. The animals were fed a conventional 41B cube diet (Pilsbury Ltd., Birmingham) and received tap water *ad libitum*. Mice were allowed to habituate in the quiet experimental room at least 24 hours prior to experimentation. The experimental room was maintained at 21°C (± 2°C), relative humidity of 50-60%, and the animals were exposed to 12 hour light-dark cycle (light; 08:00-20:00 hours).

4.2. Injection Techniques

Subcutaneous (s.c.) injection:

Injection was given to the mice via the loose skin at the back of the neck, and injection volume was 10.0ml/kg.

Intraperitoneal (i.p.) injection:

Injection was performed by insertion of the hypodermic needle into the abdominal wall towards the diaphragm. Care was taken not to penetrate too deeply in order to avoid damage to the internal organs. The injection volume used was 10ml/kg.

4.3. Procedures for head twitch analysis in mice

Experimentally naive mice from the same stock cage were placed in sawdust lined glass observation aquaria (dimensions; 33 x 23 x 20 cm), in groups of 4 animals per aquarium (Boulton and Handley, 1973), 60 minutes prior to induction of head twitch behaviour. The mice were injected with the test drug (or 0.9% saline vehicle) at the relevant pretreatment time prior to head twitch determination (details provided in Chapter 4). A submaximal dose of DOI (0.5mg/kg i.p.), determined via a dose response curve (see: Figure XX), was then administered 5 minutes prior to measurement of head-shake (HS) frequency. The mice were recorded for a 10 minute period (except for dose-response experiment), using a Panasonic System Camera WV-KT 115E with integral microphone linked to a Panasonic Video Cassette Recorder NV-FS90B. The number of HS exhibited by each animal over this period was subsequently ascertained by video analysis. At least 6 mice received each dose of a particular test agent, and where possible the experimenter remained unaware of the treatment adminstered to the mouse.

For spontaneous HS determinations the procedure differed only in the fact the mice were not pretreated with DOI, and the HS observation period was 30 minutes due to the much lower frequency of these shaking behaviours.

5. Statistical analysis of data

All mean values quoted within this thesis are expressed as the arithmetic mean plus or minus one standard error (SEM). In graphs containing mean values, vertical bars are used to represent SEM's. Unless otherwise stated, all probabilities are values for two-tailed probability. In cases of a directional hypothesis, one-tailed probabilities were used.

The differences between treatment groups were compared using Student's t-test. Analysis of variance was used for the comparison of parametric data from more than two groups, as indicated in the relevant chapters.

Correlation of parameteric data utilised the Pearson product-moment correlation coefficient, and the Spearman correlation coefficient was used for assessing correlations which involved rating scales.

Comparison of non-parametric frequency data was performed using the Chi^2 test.

Statistical significance was set at a probability level of α=0.05.

The data was analysed as described above and in the relevant results chapters using the Statistical Package for Social Sciences (SPSS).

6. Drug and reagent sources and vehicles used

All HPLC solvents were HPLC grade. All other chemicals were of the highest commercial grade available.

6.1. Drugs

Anthranilic acid	Sigma
DOI ((±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane)	RBI
(±)-epibatidine dihydrochloride	RBI
3-Hydroxyanthranilic acid	Sigma
5-hydroxy-3-indoleacetic acid	Sigma
3-Hydroxykynurenine	Sigma
5-Hydroxytryptamine hydrochloride	Sigma
(±)-Isoprenaline hydrochloride	Sigma
Kynurenic acid	Sigma
L-kynurenine sulphate	Sigma
Mecamylamine hydrochloride	Sigma
(−)-Nicotine	Sigma
Quinolinic acid	Sigma
L-tryptophan	Sigma
Xanthurenic acid	Sigma

For behavioural experiments DOI, nicotine, epibatidine and mecamylamine were all dissolved in saline (0.9% sodium chloride) . All doses are expressed as the free base of the drug.

6.2. Reagents

Acetic acid (glacial)	Fisher
Acetonitrile (HPLC grade)	Fisher
Ammonium sulphamate	Sigma
Amyl alcohol	BDH
(EDTA) Disodium-ethylene diamine	BDH
Hydrochloric acid	Fisher
Methanol (HPLC grade)	Fisher
N.E.D. (naphthylethylene diamine dihydrochloride)	Sigma
1-Octylsulphonic acid	BDH
Orthophosphoric acid	Fisher
Potassium dihydrogen orthophosphate	Fisher
Perchloric acid (70% v/v)	BDH
Sodium Acetate	Fisher
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	Fisher
Sodium hydroxide	Fisher
Sodium metabisulphite	Fisher
Sodium nitrite	Sigma
Tiron (1,2-dihdroxybenzene-3,5-disulphonic acid)	BDH
Water (HPLC grade)	Fisher
Zinc acetate	Fisher

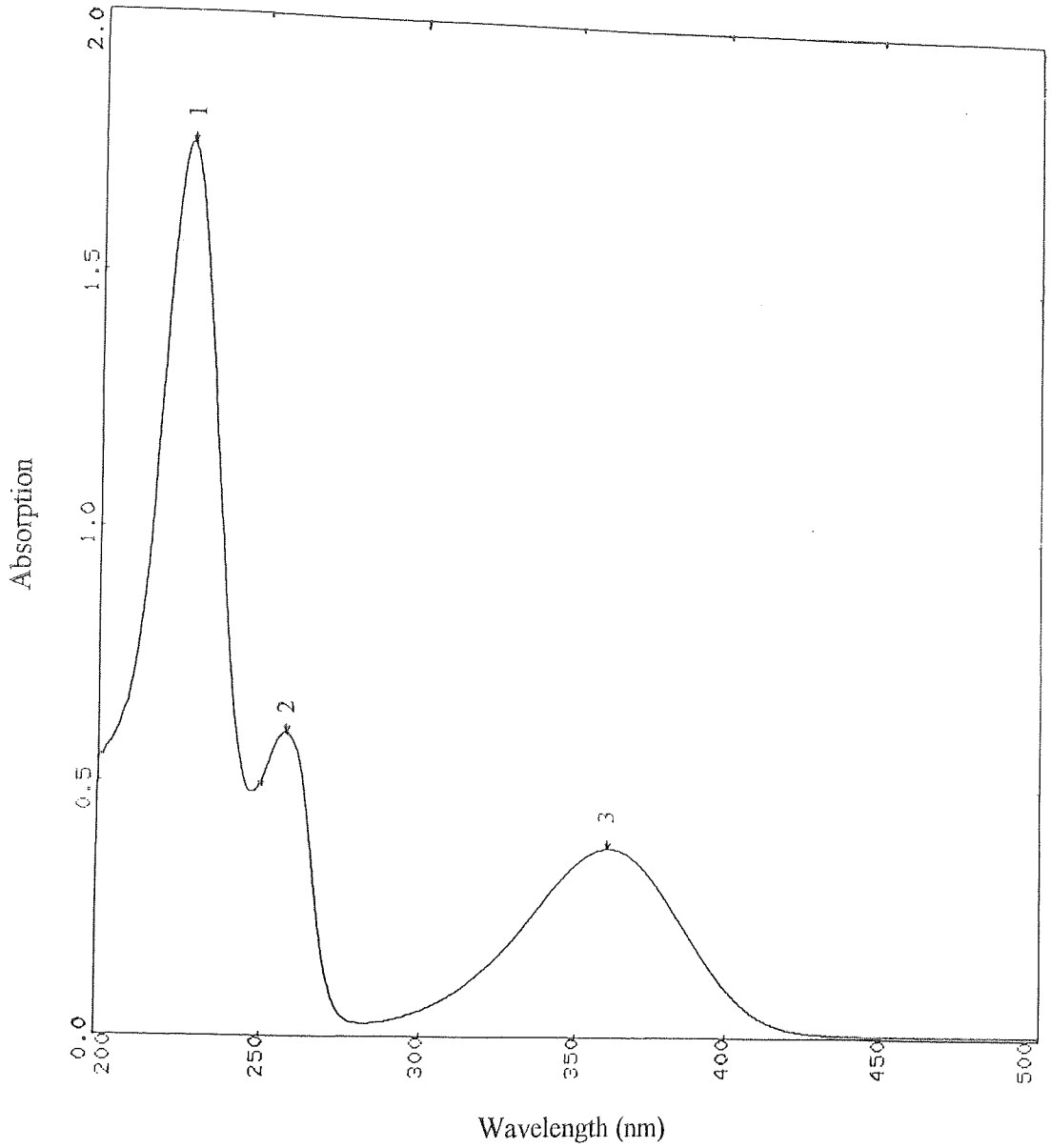

Figure I. UV absorption spectrum for a KYN (10μmolar) standard solution. Wavelength range tested: 200 to 500nm.

Peak wavelengths detected:

	1	2	3
Wavelength (nm)	227.4	257.6	360.2
absorption	1.749	0.599	0.376

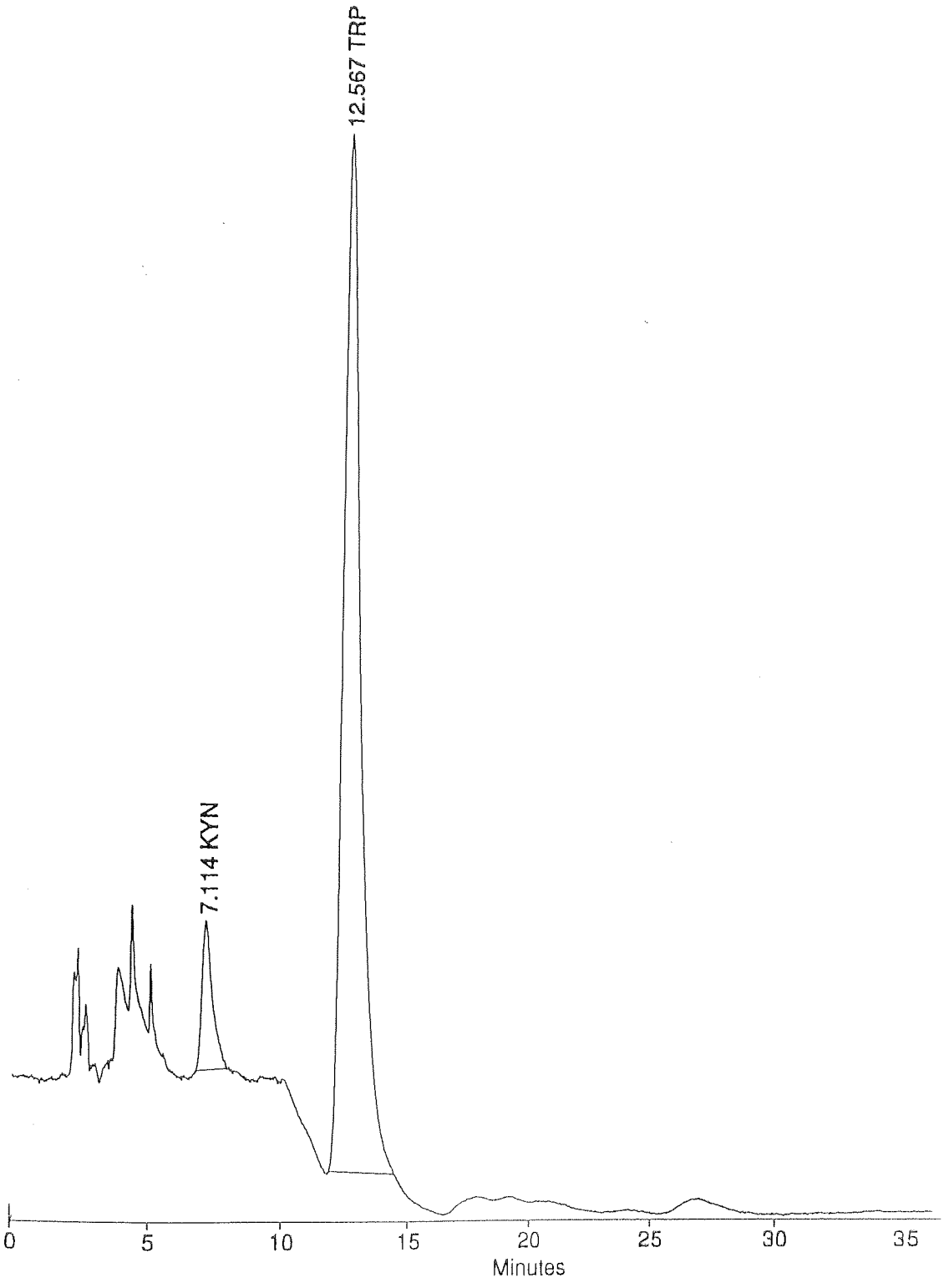

Figure II. Chromatogram showing a typical KYN (as well as TRP) peak in an extract of human plasma, measured using UV detection.

Detector settings:			
Time (mins)	0	10	36
Wavelength (nm)	360	254	254

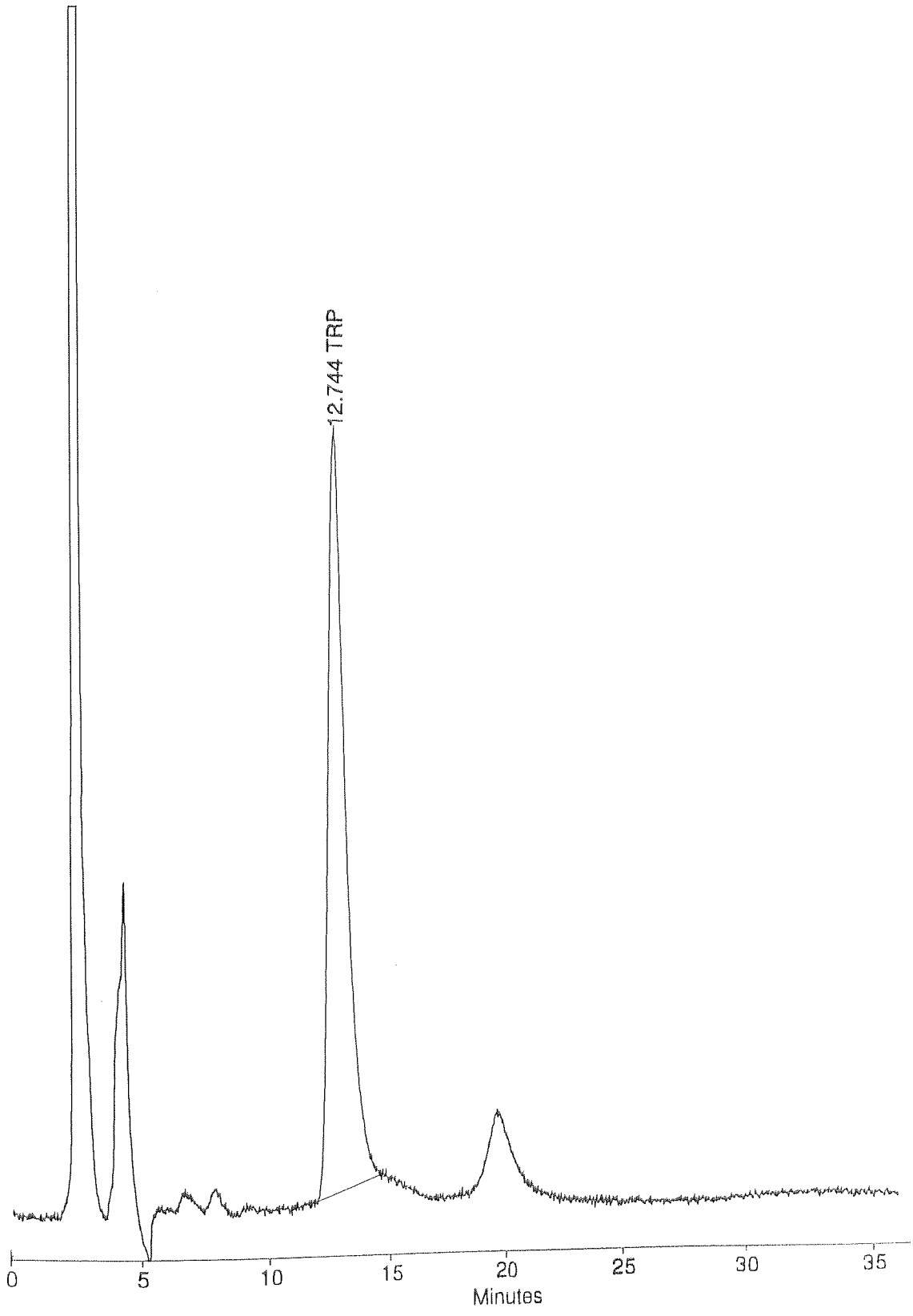

Figure III. Chromatogram showing a typical TRP peak in an extract of human plasma, measured using a fluorescence detector (excitation wavelength, 304nm; emission wavelength, 400nm).

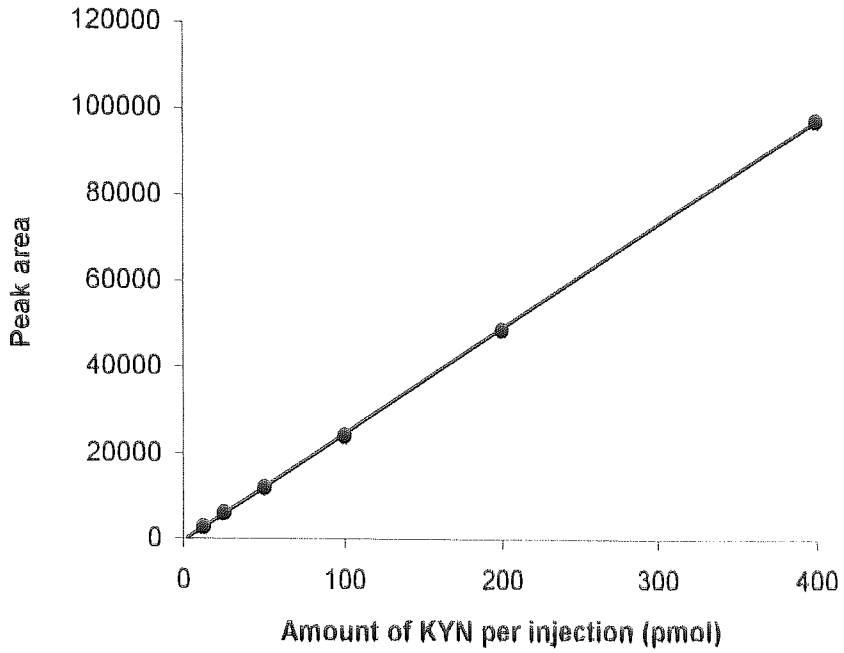

Figure IV. Relationship between amount of KYN (standard) injected onto the HPLC and peak area, measured using UV detection (absorption wavelength: 360nm).

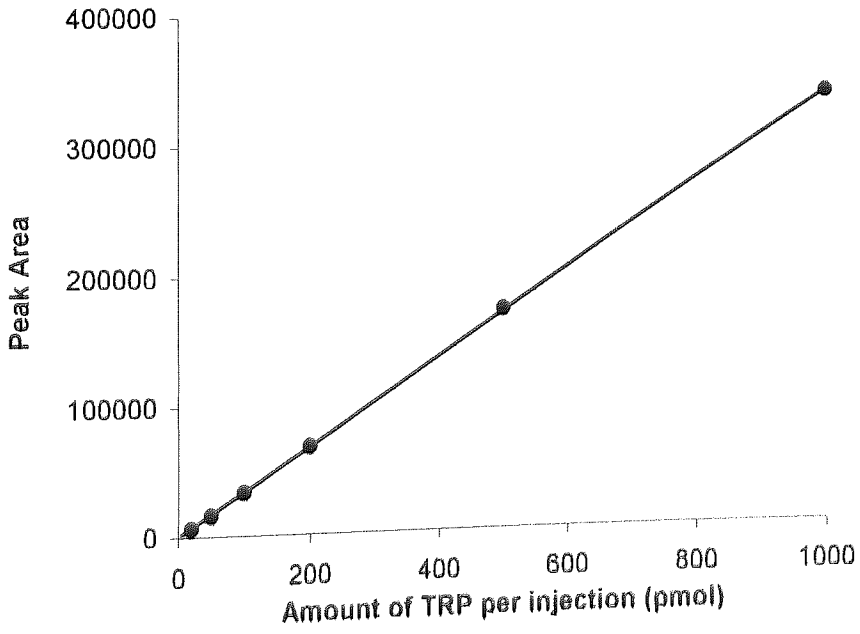

Figure V. Relationship between amount of TRP (standard) injected onto the HPLC and peak area, measured via a fluorescence detector (excitation wavelength, 304nm; emission wavelength, 400nm).

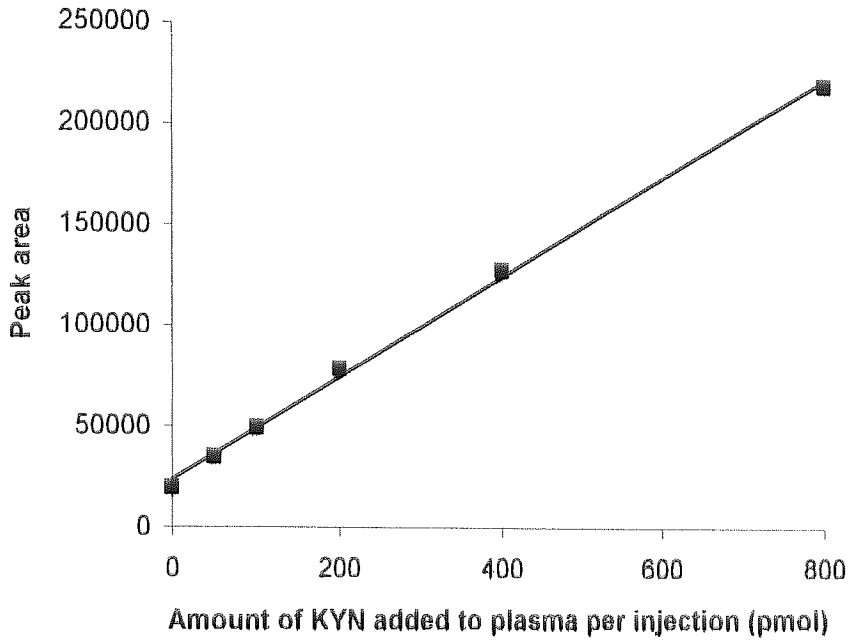

Figure VI. Graph to show linear increase in peak area after the addition of varying amounts of KYN standard to individual aliquots of a plasma sample (measured via UV detection at 360nm).

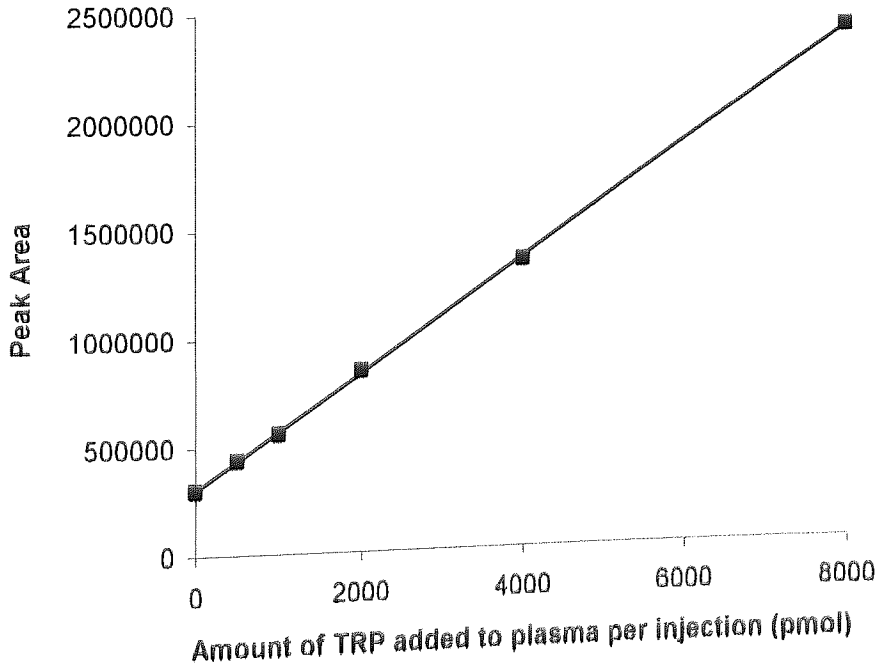

Figure VII. Graph to show linear increase in peak area after the addition of varying amounts of TRP standard to individual aliquots of a plasma sample (measured using fluorescence detection: excitation wavelength, 304nm; emission wavelength, 400nm).

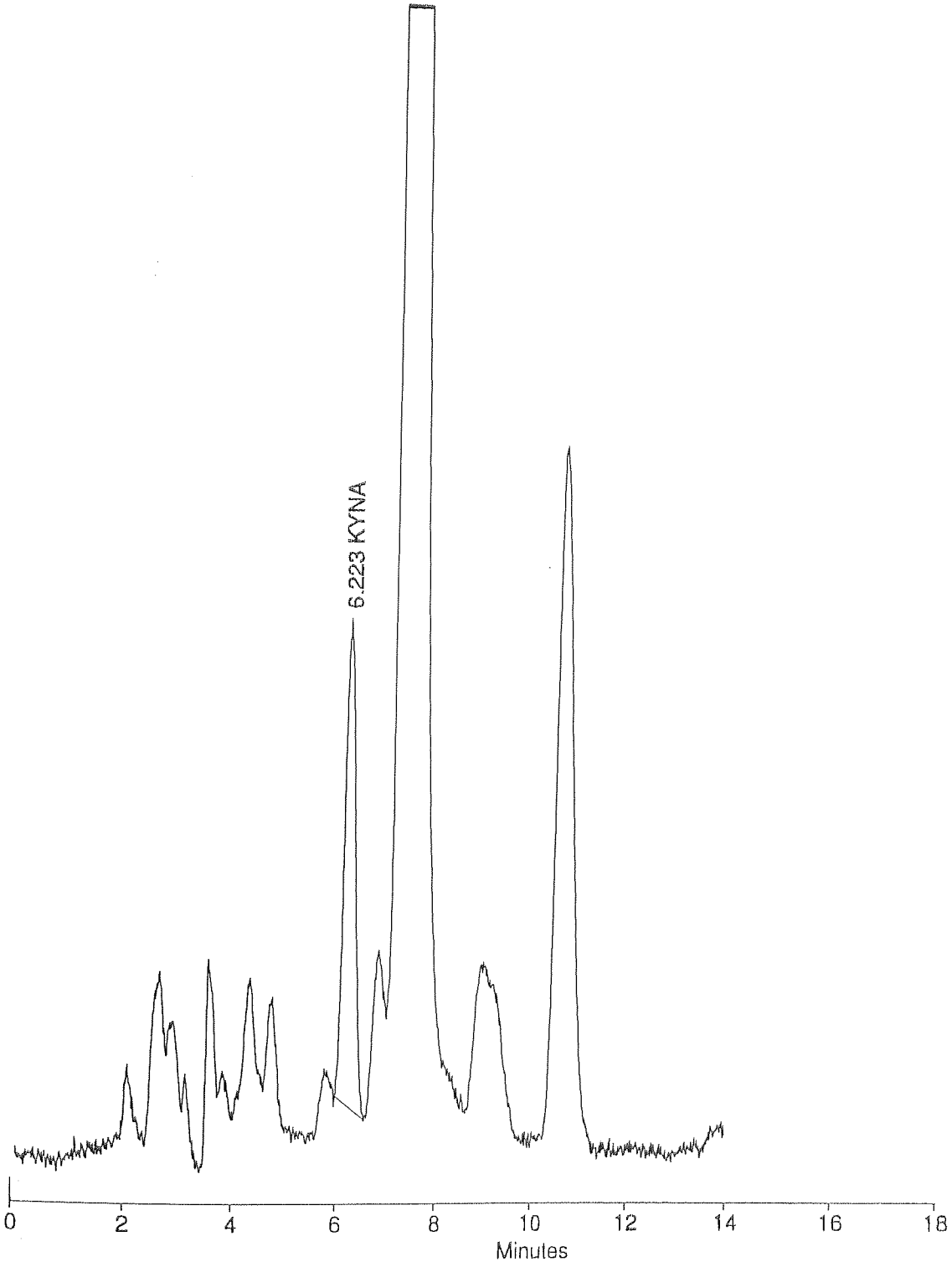

Figure VIII. Chromatogram showing a typical KYNA peak in an extract of human plasma, measured using a fluorescence detector (excitation wavelength, 254nm; emission wavelength, 398nm), with zinc acetate employed as a post-column reagent.

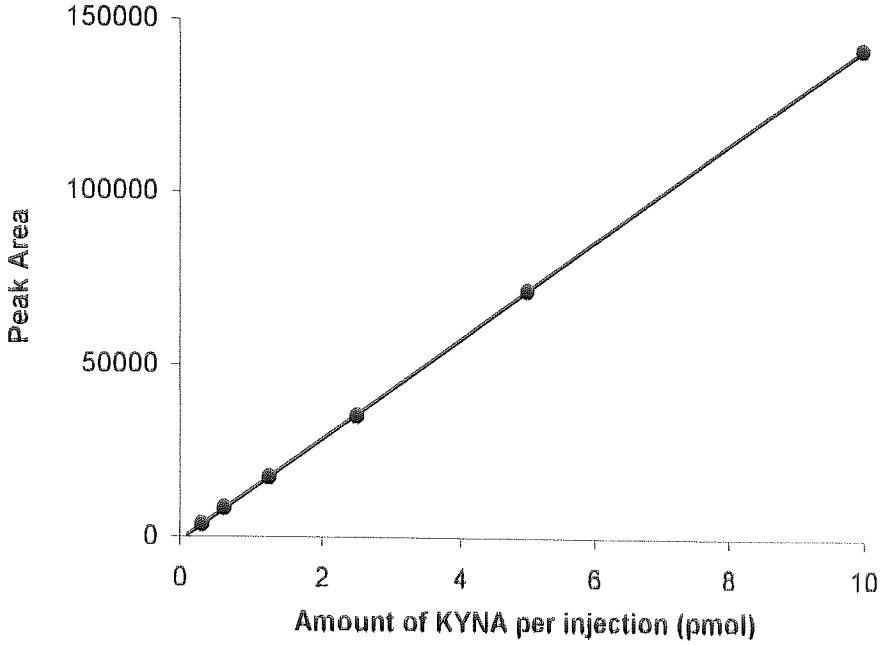

Figure IX. Relationship between amount of KYNA (standard) injected onto the HPLC and peak area, measured via a fluorescence detector (excitation wavelength, 254nm; emission wavelength, 398nm), with zinc acetate as a post-column reagent.

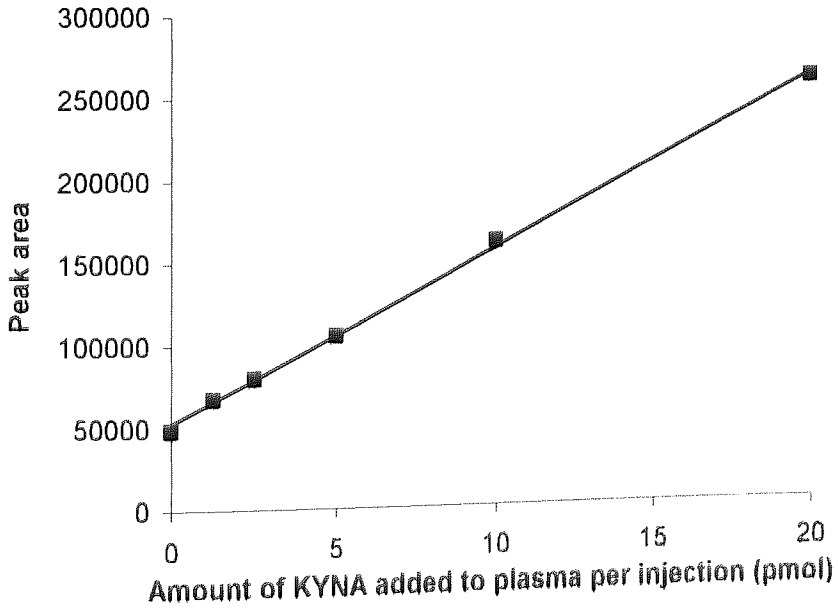

Figure X. Graph to show linear increase in peak area after the addition of varying amounts of KYNA standard to individual aliquots of a plasma sample (measured via fluorescence detection: excitation wavelength, 254nm; emission wavelength, 398nm) with zinc acetate as a post-column reagent.

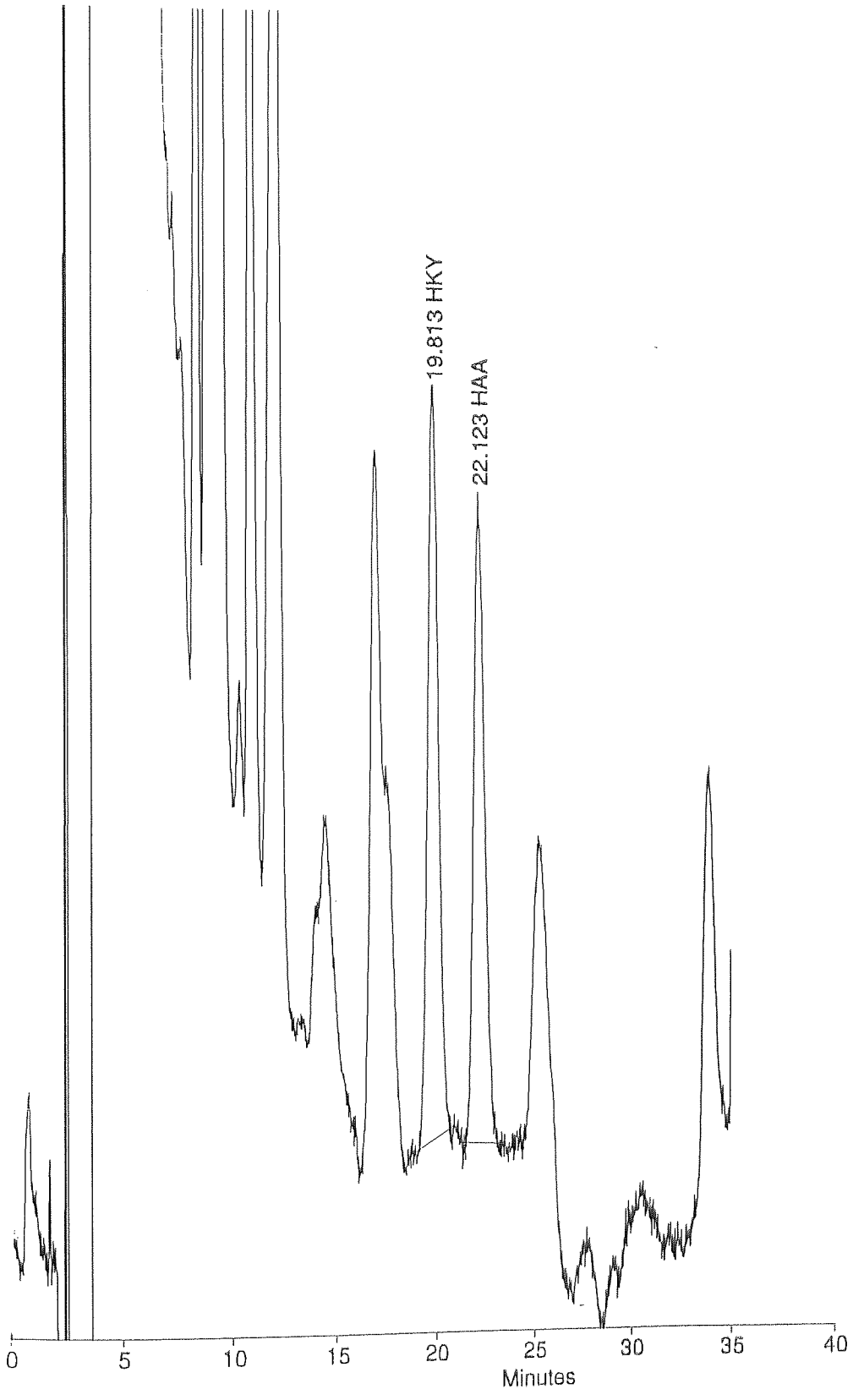

Figure XI. Chromatogram showing typical HKY and HAA peaks in an extract of human plasma, measured using ECD (working potential; +0.60V).

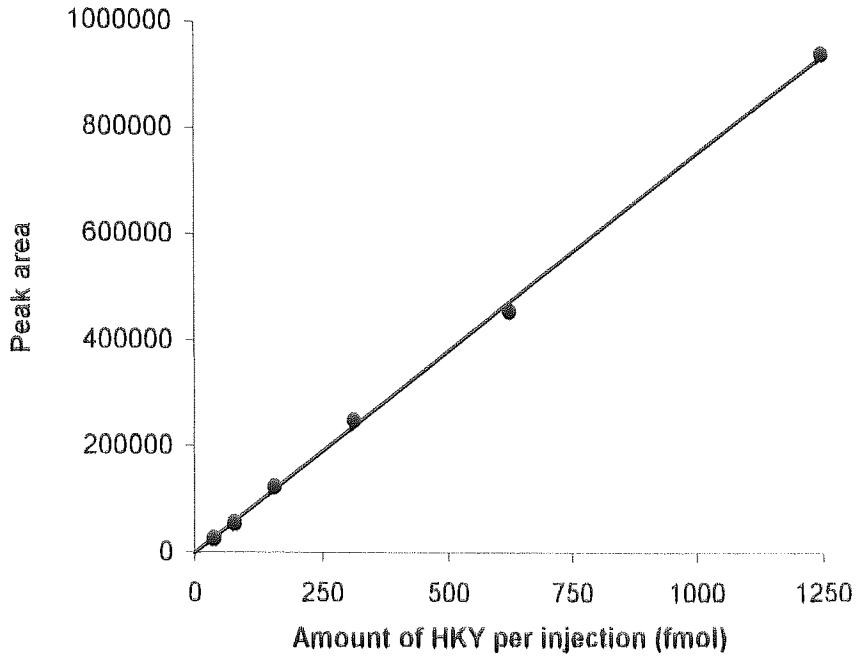

Figure XII. Relationship between amount of HKY (standard) injected onto the HPLC and peak area, measured using ECD (working potential; +0.60V).

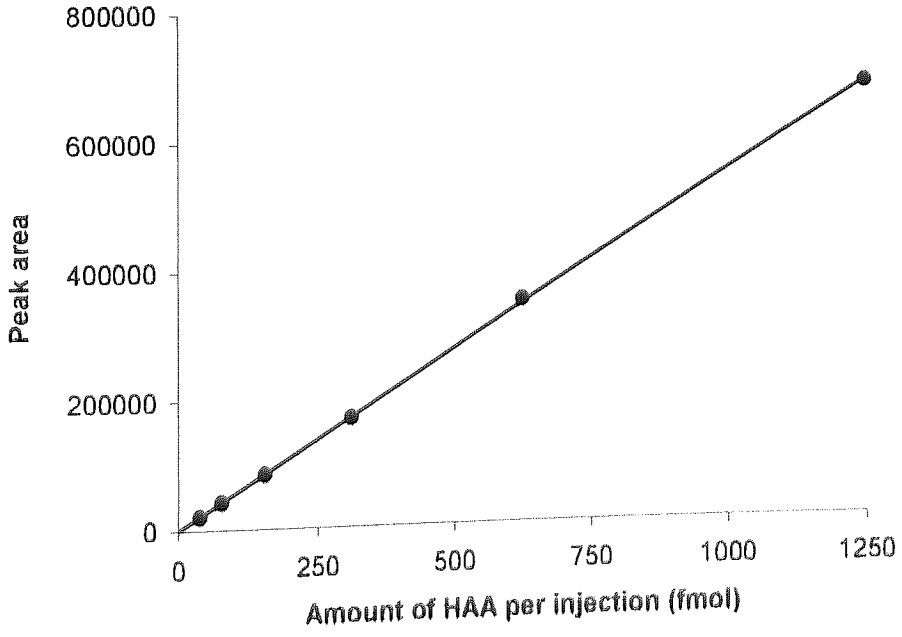

Figure XIII. Relationship between amount of HAA (standard) injected onto the HPLC and peak area, measured using ECD (working potential; +0.60V).

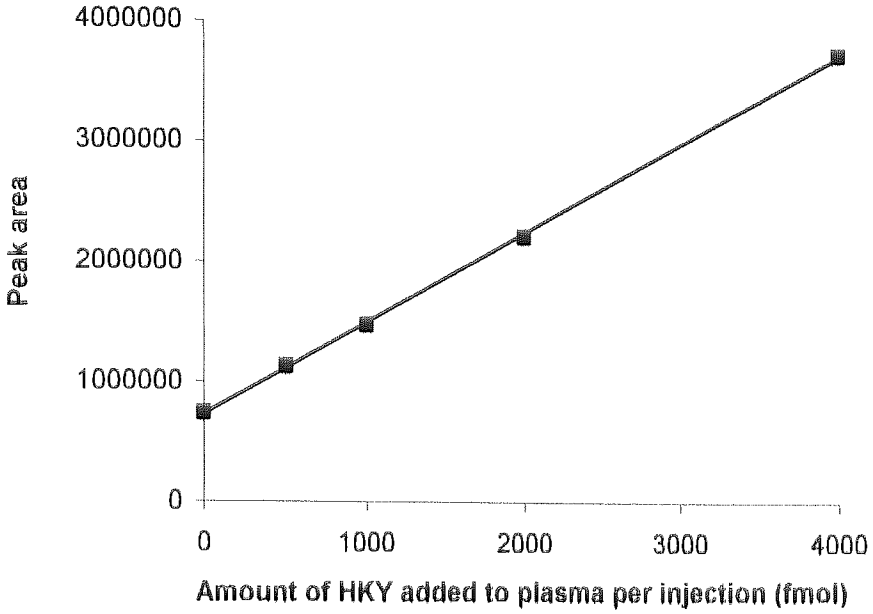

Figure XIV. Graph to show linear increase in peak area after the addition of varying amounts of HKY standard to individual aliquots of a plasma sample (measured via ECD: working potential +0.60V).

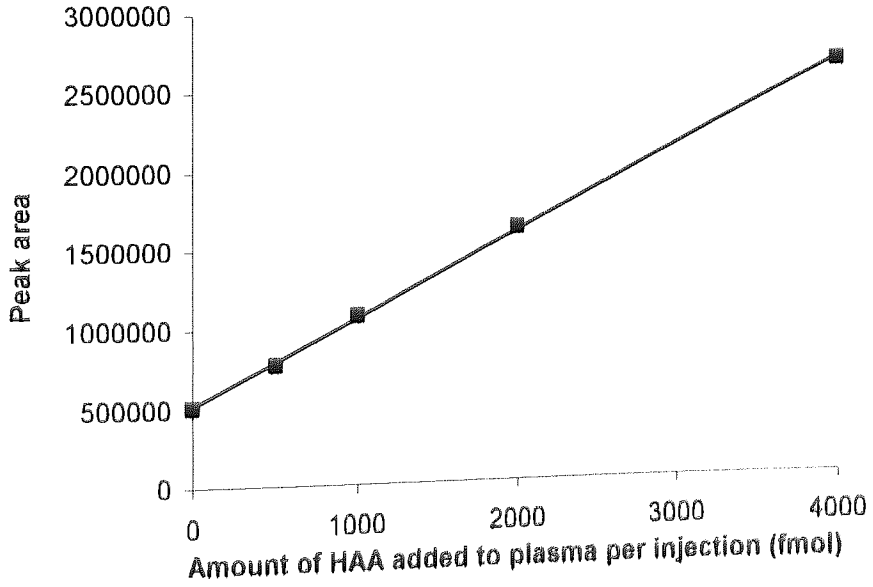

Figure XV. Graph to show linear increase in peak area after the addition of varying amounts of HAA standard to individual aliquots of a plasma sample (measured via ECD: working potential +0.60V).

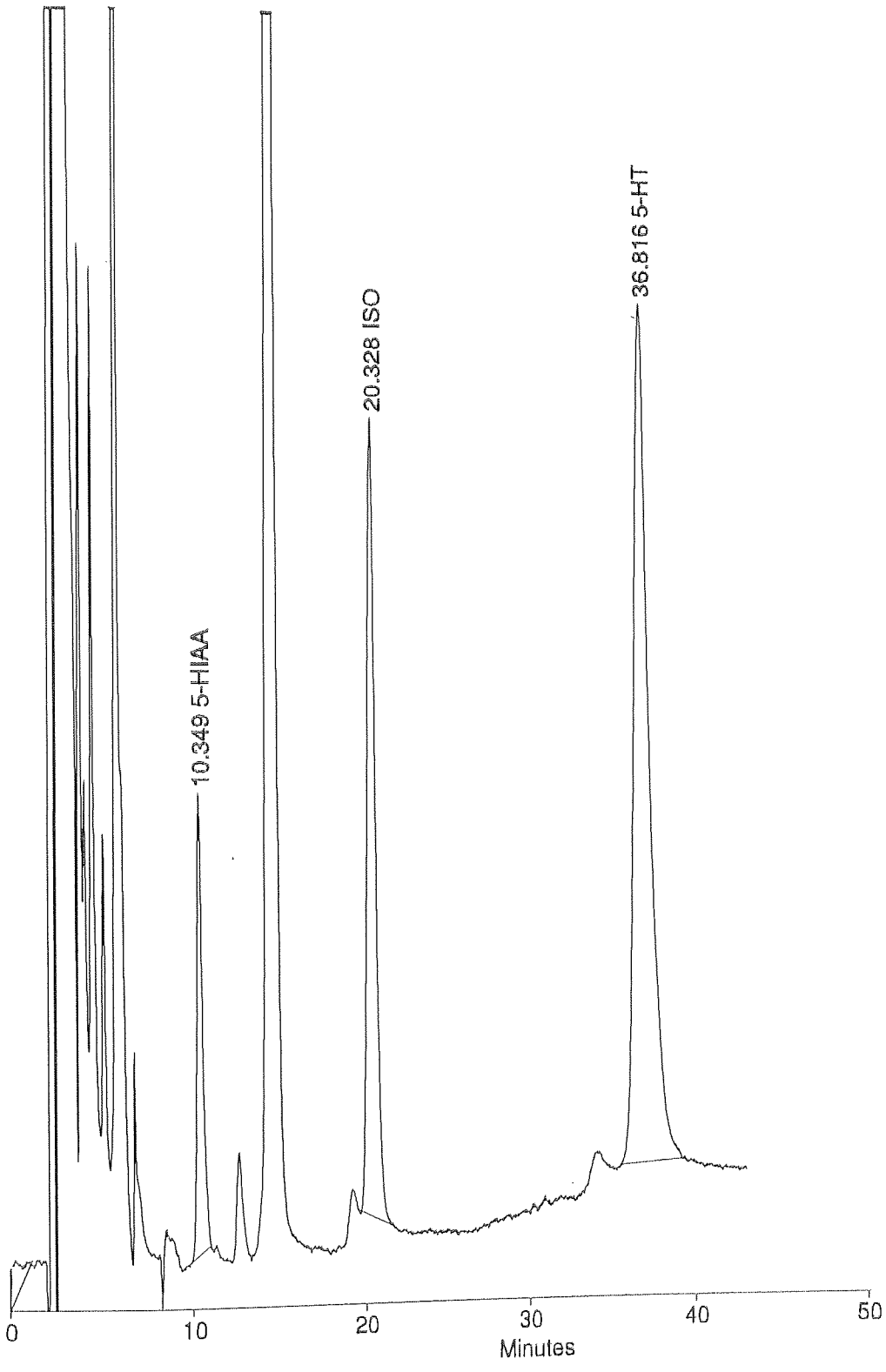

Figure XVI. Chromatogram showing typical 5-HT, 5-HIAA and isoprenaline (internal standard) peaks in an extract of mouse brain, measured using ECD (working potential; +0.70V).

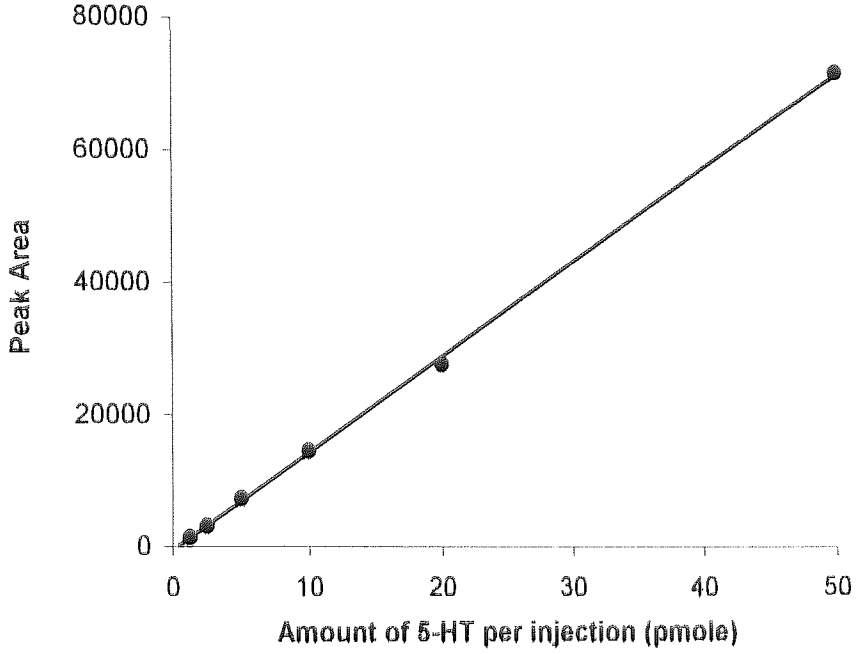

Figure XVII. Relationship between amount of 5-HT (standard) injected onto the HPLC and peak area, measured using ECD (working potential; +0.70V).

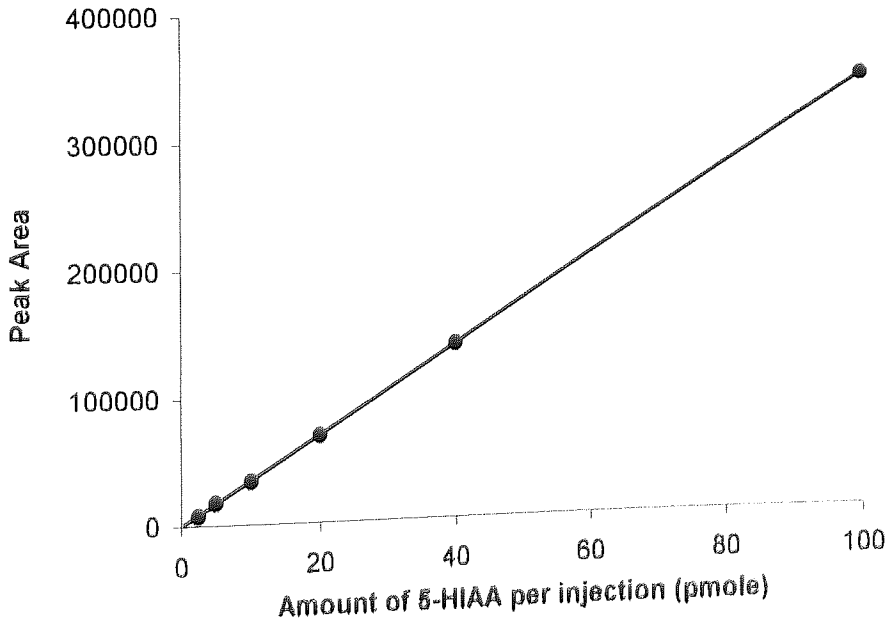

Figure XVIII. Relationship between amount of 5-HIAA (standard) injected onto the HPLC and peak area, measured using ECD (working potential; +0.70V).

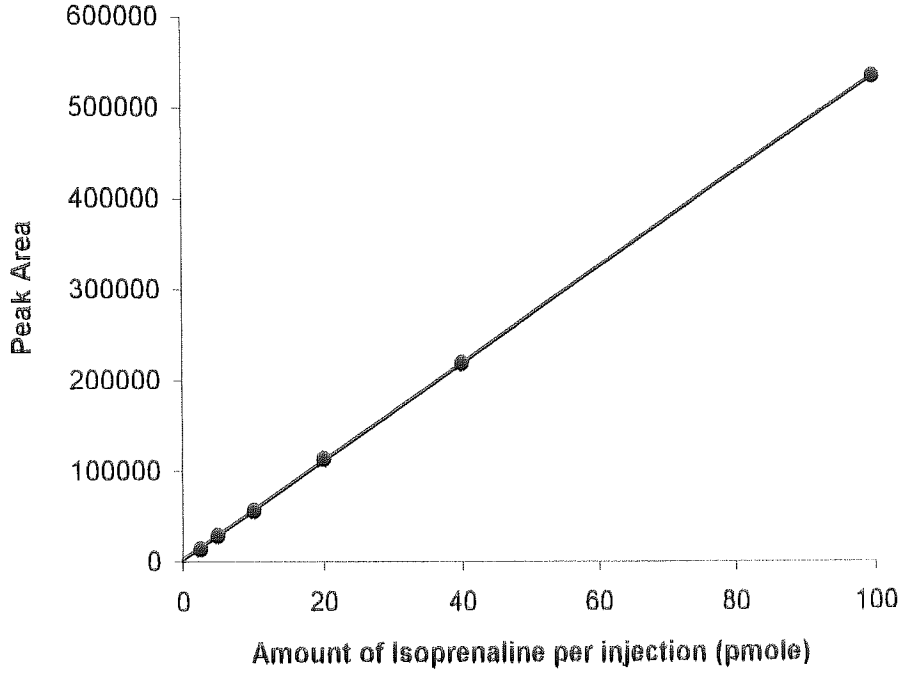

Figure XIX. Relationship between amount of isoprenaline (standard) injected onto the HPLC and peak area, measured using ECD (working potential; +0.70V).

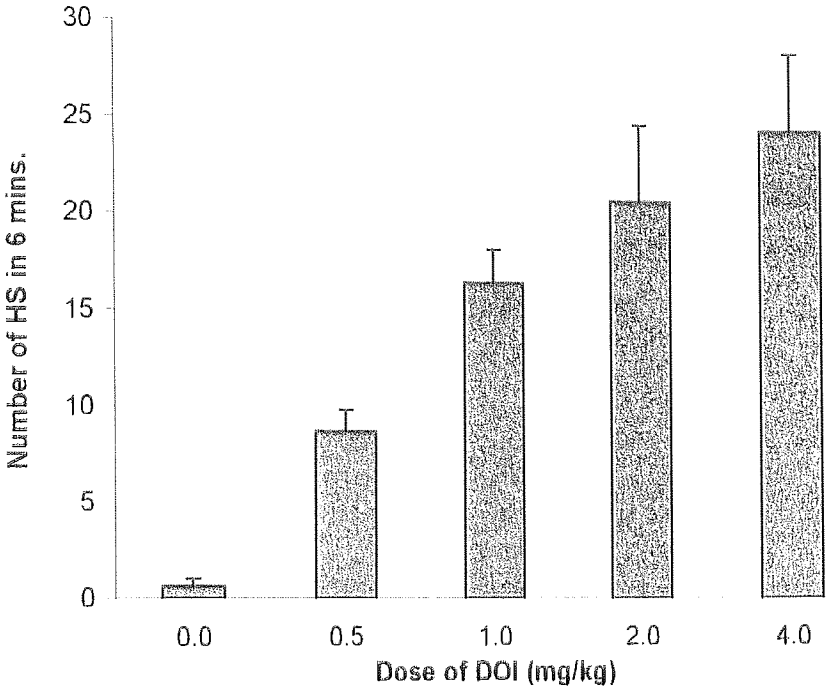

Figure XX. DOI Dose-response graph for head-shakes in mice.

sample sizes for each dose of DOI: 0 mg/kg (saline vehicle), n=5; 0.5 mg/kg, n=11; 1.0 mg/kg, n=11; 2.0 mg/kg, n=6; 4.0 mg/kg, n=6.

CHAPTER 1

THE EFFECTS OF DIETARY STATUS AND TIME OF DAY ON KYNURENINE PATHWAY VARIABLES IN TS PATIENTS AND HEALTHY CONTROLS.

CHAPTER 1

Introduction

The kynurenine pathway is the principal route of TRP metabolism in the body, and plasma KYN levels give a gross index of the activity of this pathway (refer to: General Introduction). A small percentage of dietary TRP is also utilised in the formation of 5-HT. In the liver, the first enzyme and rate limiting step in the kynurenine pathway, is cortisol-inducible TDO, while in extrahepatic tissues (including brain) the corresponding enzyme is cytokine-inducible IDO (see: General Introduction). A widely accepted marker of cell mediated immunity is neopterin, and like KYN, its production is stimulated by cytokines via a series of enzymatic reactions (Wachter *et al.*, 1992).

There are conflicting reports with regard to alterations in TRP metabolism and abnormalities in the 5-HT system in TS. An initial study by Comings (1990a), in which all subjects were fasting, found that unmedicated TS patients exhibited reduced whole blood TRP and 5-HT compared with normal controls. In a second study, where dietary factors were not controlled, there was a decrease in TRP in the TS group, but no difference in whole blood 5-HT concentration was detected (Leckman *et al.*, 1984). In contrast, Dursun *et al.* (1994a) found no change in plasma TRP between 7 fasting TS patients and 10 matched controls, but a significant elevation in plasma KYN concentration was shown by the TS group, although the mechanism of this change was unclear.

In a more recent investigation, Rasmusson and coworkers (1997) could find no evidence of worsening of tic, obsessive-compulsive or mood symptoms in response to TRP depletion in TS subjects. The authors therefore inferred that reductions in plasma TRP, and the consequential decreases in CNS TRP and 5-HT concentrations (Moja *et al.*, 1989), do not influence TS symptoms. However, the design of the study could have been significantly improved by also including non-TS control subjects (rather than using TS patients only), to support their interpretation of the data.

In mammals, approximately 90% of all TRP molecules in blood are loosely bound to the plasma protein albumin (Mcmenamy and Oncley, 1958), with the remainder circulating in the free form. TRP appears to be the only amino acid to distribute itself

in this way between a bound and a free form. Accordingly, since only unbound TRP enters the brain, it has been suggested that the size of the free TRP pool in the blood is an important determinant of brain TRP uptake (Knott and Curzon, 1972). On the other hand, studies have shown that most of the TRP molecules are weakly associated with albumin and can readily dissociate from the protein molecules, so that both free and bound TRP in blood are likely to influence the amount of TRP transported into the brain (Yuwiler *et al.*, 1977).

TRP crosses the blood-brain barrier from the periphery via a saturable, competitive carrier-mediated system, which is shared with other large neutral amino acids (LNAAs), including valine, leucine, isoleucine, tyrosine and phenylalanine and possibly methionine and threonine (Fernstrom, 1983). Brain TRP levels thus increase when blood TRP levels rise, or blood levels of one or more of the other LNAA competitors fall. Conversely, when blood TRP levels fall or blood concentrations of the other LNAAs rise, TRP levels in the brain are reduced. Consequently, it has been proposed that the total plasma TRP/LNAA ratio provides the best estimate of TRP influx into the brain (as well as brain 5-HT concentrations), as opposed to total plasma TRP (Fernstrom, 1983, Moja *et al.*, 1989). However, Fernstrom (1983) acknowledges that these blood ratios do not take into account the unique affinity of each LNAA for the brain transport carrier. Nevertheless, this quotient could provide an indication of changes to KYN brain levels and its other pathway metabolites.

Given the ability of the diet to modify the balance of various nutrients in the body, research has been conducted to examine how diet may affect brain serotonergic parameters, either by supplying the neurotransmitter precursor TRP in quantities which alter the plasma pool size, or by changing plasma concentrations of the LNAAs which compete with TRP for the carrier-mediated transport mechanism into the brain (Ashley *et al.*, 1985). These studies have demonstrated that the time of day that a meal is consumed may be important, with breakfast meal composition appearing to exert a greater influence on plasma TRP/LNAA ratio than evening meal (Ashley *et al.*, 1985).

In spite of these potential dietary effects on biochemical pathways, it should be borne in mind that TRP is a nutrient normally provided to the body (and brain) in modest quantities, and so it has been questioned whether large scale changes in the formation

and release of 5-HT (or other biological compounds) will occur (Fernstrom, 1983). In addition, the conversion of TRP to 5-HT takes place only in those cells that normally make it, and probably at a rate that is not especially rapid, compared for example to 5-HTP, which after administration is swiftly decarboxylated to 5-HT by any cell in the body (Fernstrom, 1983).

The principle objectives of this study were twofold. Firstly, we were attempting to replicate the previously reported increase in plasma KYN in TS patients compared with normal controls (Dursun *et al.*, 1994a), and to look at the most likely source of such changes. Secondly, we felt it necessary to investigate the effects of diet and time of day on individual kynurenine pathway parameters. The following experiment was thus designed primarily as a pilot study, serving to identify specific dietary restrictions and diurnal factors, which may need to be addressed prior to the much larger cross-sectional study (see: Chapter 2).

Total plasma TRP was measured to reflect the amount of TRP available as a KYN precursor, but may also represent a source of other further metabolites.

Additional methods

All control candidates were selected on the basis of the criteria specified in the General Methods, and were recruited from Aston University staff and students. TS subjects were obtained via the TS clinic (Queen Elizabeth's Psychiatric Hospital, Birmingham).

Dietary status of subjects and controls in pilot study

In all cases, volunteers were asked to starve themselves from 00:00 hrs on the night preceding the study. All candidates were also asked to refrain from nicotine intake during the day of the study, following evidence suggesting a role for the nicotinic cholinergic system in TS (see: General Introduction). In addition, intake of caffeine-containing products was prohibited, due to reports of increased KYN concentration in plasma, resulting from an anxiety-inducing dose of caffeine (Orlikov and Ryzov, 1991). Finally, candidates were not permitted to take any aspirin products from midnight prior to the study, since it is known to interfere with the binding of TRP to plasma albumin (McArthur and Dawkins, 1969).

On arrival at the clinical study venue, the TS subject (TS; n=11) and normal control (NC; n=6) groups were subdivided into a free-feeding (FF) (TS_{FF} n=6; NC_{FF} n=3) or controlled feeding (C) (TS_C n=5; NC_C n=3) group. Once the initial blood samples had been taken from each volunteer (at 09:30 hrs), the FF group was permitted to eat and drink without restriction throughout the day (with the exception of the products previously detailed). The C group was provided with a standard breakfast consisting of two Weetabix (plus milk and sugar) and a glass of fruit juice. A standard meal consisting of a milkshake was also provided for their lunch (about 13:00 hrs). Between breakfast and lunchtime members of the C group were allowed to consume fruit juice and water only. Venous blood samples were taken aseptically at 09:30, 11:00, 12:00, 14:00 and 15:00 hrs and plasma was subsequently obtained and stored as outlined in the General Methods section.

Measurement of Biochemical variables

Plasma concentrations of KYN, TRP, KYNA (all quantified by HPLC) and cortisol (radioimmunoassay) were measured for the time categories 09:30, 11:00, 14:00 and 15:00 hrs, and plasma neopterin was determined for the 09:30 and 15:00 hrs time periods only (for details of procedures refer to: General Methods). The plasma obtained at 12:00 hrs was used exclusively for determining plasma KYN concentrations via the method of Joseph and Risby (1975) (see: General Methods). This latter method was employed by Dursun *et al.*, (1994a) for measurement of KYN in their plasma samples.

At the 09:30 hrs time interval it was possible to compare the biochemical variables of the TS and control groups directly, since the division of candidates into their dietary categories had not yet been instigated.

Statistics

All values quoted are means ± SEM. A Student's *t*-test was used to determine differences between two groups of treatment. Difference between multiple groups were analysed by three factor ANOVA as a function of subject, diet and gender. Biochemical data were also compared where appropriate using correlational analysis.

Because of the sheer number of individual ANOVA results tables yielded by our data, only those providing statistically significant findings have been included in the list of tables for this chapter.

Results

Plasma KYN, TRP, KYNA, Neopterin and Cortisol concentrations in TS patients and controls at 09:30 hours

There was no significant difference between TS subjects and controls for any of the biochemical variables in the plasma samples collected at 09:30 hrs following overnight fast (see: Table 1.1). Perhaps the most interesting observation, however, relates to a non-significant trend towards decreased plasma TRP (NC=58.9 ± 2.6; TS=50.4 ± 3.4, μmol/L; p=0.11) and KYN (NC=1.40 ± 0.15; TS=1.10 ± 0.10, μmol/L; p=0.11) in the TS subject group.

Plasma KYN at 09:30, 11:00, 14:00 and 15:00 hrs

No statistically significant effect of subject, diet or gender on plasma KYN concentrations was detected for any of the time parameters. The 3-way ANOVA for KYN at 11.00 hrs (see: Table 1.2) has been included to illustrate this result. The data for subject and control groups at each time interval is also represented in Figure 1.1.

Plasma TRP at 09:30, 11:00, 14:00 and 15:00 hrs

A weak subject main effect ($F_{(1, 16)}$=5.42; p<0.05) on plasma TRP concentrations for the 09:30 hrs time samples was shown by a 3-way ANOVA (Table 1.3). Although a trend towards decreased TRP was exhibited by the TS group at this sample time, the difference was not significant (see: Table 1.1 and Figure 1.2). No further significant effects of subject, diet or gender on plasma TRP levels were observed at any other times studied.

Plasma KYNA at 09:30, 11:00, 14:00 and 15:00 hrs

A weak main subject effect ($F_{(1, 16)}$=5.38; p<0.05) on plasma KYNA was observed at 15:00 hrs (see: Table 1.4), and a *t*-test verified that the control group had significantly higher plasma KYNA levels compared with TS subjects (p<0.05) for this

sample time (refer to: Figure 1.3). However, given that there were no other statistically significant effects of subject, diet or gender for any other plasma sample times, the reliability of this difference is questionable.

Plasma cortisol at 09:30, 11:00, 14:00 and 15:00 hrs

A 3-way ANOVA of cortisol concentrations indicated a weak diet main effect ($F_{(1, 16)}=7.55$; $p<0.05$) and a subject × gender interaction ($F_{(1, 16)}=6.35$; $p<0.05$)(see: Table 1.5). However, as the cell mean plots show (Figure 1.4), these effects appear to arise predominantly via single (female) outlier, who exhibited a plasma cortisol reading far in excess of any other person in the study. The results for subsequent sample times were suggestive of a persistent distortion of the data originating from this same candidate (*i.e.* all ANOVA tables showed similar findings to that given in Table 1.5), who consistently gave higher cortisol readings than any other individual. Therefore, in view of the small sample size, and even smaller subgroups, these effects were considered unreliable.

The graph showing mean plasma cortisol against sample time for TS subjects and controls (see: Figure 1.5), illustrates a gradual reduction of mean plasma cortisol levels throughout the course of the day. Just how important the time of day (when biological samples are acquired) is to the design of such studies is clear from this plot, since differences in biochemical variables between sample groups could become distorted, either as a direct result of this diurnal variation, or via indirect effects.

Plasma neopterin at 09:30 and 15:00 hrs

There was no statistically significant effect of subject, diet or gender on plasma neopterin levels for the 09:30 or 15:00 hrs time intervals (ANOVA tables not shown). Variation in plasma neopterin for TS subjects and controls at 09:30 and 15:00 hrs is shown in Figure 1.6.

Comparison of plasma KYN values at 11:00 hrs and 12:00 hrs using HPLC and the Joseph and Risby (1975) methods respectively

The KYN concentrations obtained using the two methods were not significantly correlated, although KYN data measured by HPLC (for the various time parameters)

demonstrated a close correlation (refer to: Tables 1.6 and 1.7). Significantly, however, the data obtained via the Joseph and Risby (1975) procedure, in agreement with the KYN values measured by HPLC, did not detect any change between TS patients and controls. It is also noteworthy, that the plasma KYN values obtained via the Joseph and Risby (1975) method, were recorded at the very limit of sensitivity.

Discussion

Contrary to the findings of Dursun *et al.* (1994a) we found no change in plasma KYN levels between fasting TS patients and controls (by analysis of the 09:30 hours samples). A possible reason for this discrepancy might be provided by differences in the methods of KYN measurement. The Joseph and Risby (1975) procedure was used by Dursun and colleagues, which involved a series of extraction steps for the removal of proteins from the plasma, and subsequent formation of an azodye complex (via diazotisation and coupling reactions), which is measured by UV absorption (full details in General methods). Owing to the large volumes of plasma required for this assay, we were unable to quantify samples from the same time interval using both HPLC and the Joseph and Risby (1975) method. Nevertheless, in an attempt to equate the contrasting results from this study with those reported by Dursun *et al.* (1994a), the latter procedure was employed for determining KYN in plasma samples obtained at the 12:00 hrs time. Subsequent comparison with the KYN values at 11:00 hrs (measured by HPLC), which represents the closest time parameter to 12:00 hrs, indicated no statistically significant correlation between the two sets of data from our study, and importantly no difference in KYN between TS patients and controls using either method. Furthermore, none of our KYN concentrations measured by the Joseph and Risby procedure, came close to matching the high levels reported in the Dursun *et al.* (1994a) TS group. Although the sensitivity of this method is much inferior than that achieved by HPLC, procedural differences alone do not fully explain why Dursun and colleagues found KYN to be raised in all of their TS patients. In addition, a trend towards a KYN deficit was actually found within our TS cohort (at 09:30 hrs).

A second potential source for the disparity could relate to seasonal variation of certain elements, so that the time of year when blood samples are collected during

such studies may be a critical factor. A fluctuation of cortisol levels according to environmental temperature and time of year has previously been found (Handley *et al.*, 1980), which could possibly alter hepatic TDO activity, and thereby influence the levels of KYN and/or its metabolites. Since the samples for this study were collected during the hot month of July, whereas those assayed by Dursun and colleagues were gathered in the cooler October to December period (personal communication), this hypothesis remains feasible.

No significant differences in the concentrations of TRP, KYNA, neopterin or cortisol were found between the control and TS groups at the 09:30 hrs time, which thus failed to substantiate our theory of an abnormality of the kynurenine pathway in TS. Nevertheless, it may be relevant that the largest difference in plasma TRP concentration between TS subject and control groups, did occur at 09:30 hrs (Figure 1.1) although it did not reach significance. The potential importance of this finding, relates to the fact that in both reports where a reduction in TRP was reported in TS patients (Leckman *et al.*, 1984; Comings, 1990a), blood samples were collected in the early part of the morning. Therefore, had the sample size of the groups been larger, we may indeed have detected a significant difference in plasma TRP.

Our results did not provide any clear evidence for dietary influences, although the importance of time of day to these types of studies was shown by the progressive fall in cortisol at consecutive sample times for TS patients and controls (see: Figure 1.4). With reference to the apparent lack of dietary influence on the biochemical variables, the small sample numbers in each of the subgroups meant that only substantial effects are likely to have been picked up. Moreover, many of the changes in blood TRP reported in similar studies concerning dietary manipulation, refer to alterations in the TRP/LNAA ratio (Ashley *et al.*,1982; Ashley *et al.*,1985), which are not solely indicative of changes in blood TRP. On the basis of our results, we felt it would be appropriate to allow the volunteers involved in the main cross-sectional study (of Chapter 2), to eat normally on the day that blood sample were submitted (although maintaining the nicotine, caffeine and aspirin restrictions). Moreover, since some of the subjects in the larger study would need to travel considerable distances to the clinics, which would be followed by various clinical assessments, it could be late

afternoon before blood samples were obtained. This further justified our decision purely from an ethical perspective.

Table 1.1. Plasma concentrations of KYN, TRP, KYNA, neopterin and cortisol in TS subjects and healthy controls (NC) after overnight fast (*i.e.* 09:30 hrs sample time).

Abbreviations: M=male, F=female.

	Subject status	Age (yrs) Sex	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)
1	NC	31; F	0.93	50.3	41.5	3.5	15.2
2	NC	45; M	1.87	53.2	95.5	8.3	8.3
3	NC	34; M	1.29	65.3	22.8	6.8	15.0
4	NC	23; M	1.59	65.5	63.7	6.8	19.8
5	NC	22; F	1.03	58.4	28.0	4.7	49.5
6	NC	36; M	1.69	60.9	59.3	5.1	13.4
Mean		31.8 ± 4	1.40 ± 0.15	58.9 ± 2.6	51.8 ± 11.0	5.87 ± 0.71	20.2 ± 6.1
7	TS	22; M	1.08	70.1	34.6	3.4	19.4
8	TS	21; M	1.08	66.0	41.5	4.6	24.7
9	TS	37; F	1.16	43.0	42.2	5.1	10.6
10	TS	16; F	1.92	39.8	38.7	12.3	7.6
11	TS	33; M	1.07	52.7	26.5	5.2	15.2
12	TS	57; M	0.73	31.9	33.9	4.2	16.5
13	TS	35; M	1.28	57.3	47.9	5.6	16.6
14	TS	37; M	1.08	51.0	37.8	3.7	9.9
15	TS	19; F	0.67	49.2	29.8	4.3	31.6
16	TS	42; M	1.17	50.2	35.0	4.3	13.6
17	TS	33; F	0.83	43.0	49.8	6.2	10.4
Mean		32.0 ± 4	1.10 ± 0.10	50.4 ± 3.4	38.0 ± 2.1	5.35 ± 0.74	16.0 ± 2.1

Table 1.2. A 3-way ANOVA to show the effects of subject, diet and gender on plasma KYN (11:00 hrs) concentration.

Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	0.248	3	0.083	0.669	0.592
SUBJECT	0.108	1	0.108	0.871	0.375
DIET	0.001	1	0.001	0.004	0.949
GENDER	0.117	1	0.117	0.949	0.355
2-Way Interations	0.337	3	0.112	0.910	0.474
SUBJECT DIET	0.001	1	0.001	0.011	0.919
SUBJECT GENDER	0.241	1	0.241	1.956	0.195
DIET GENDER	0.053	1	0.053	0.426	0.530
3-Way Interations	0.000	1	0.000	0.000	0.996
SUBJECT DIET GENDER	0.000	1	0.000	0.000	0.996
Explained	0.611	7	0.087	0.707	0.669
Residual	1.111	9	0.123		
Total	1.722	16	0.108		

Table 1.3. A 3-way ANOVA to show the effects of subject, diet and gender on plasma TRP (09:30 hrs) concentration.

Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	574.217	3	191.406	2.832	0.099
SUBJECT	366.156	1	366.156	5.419	0.045*
DIET	0.137	1	0.137	0.002	0.965
GENDER	155.428	1	155.428	2.300	0.164
2-Way Interations	255.355	3	85.118	1.260	0.345
SUBJECT DIET	204.335	1	204.335	3.024	0.116
SUBJECT GENDER	0.044	1	0.44	0.001	0.980
DIET GENDER	59.944	1	59.944	0.887	0.371
3-Way Interations	63.444	1	63.444	0.939	0.358
SUBJECT DIET GENDER	63.444	1	63.444	0.939	0.358
Explained	1121.267	7	160.181	2.370	0.114
Residual	608.173	9	67.575		
Total	1729.440	16	108.090		

Main subject effect; *, $P<0.05$ significant difference.

Table 1.4. A 3-way ANOVA to show the effects of subject, diet and gender on plasma KYNA (15:00 hrs) concentration.

Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	687.761	3	229.254	2.889	0.095
SUBJECT	426.942	1	426.942	5.381	0.046*
DIET	2.265	1	2.265	0.029	0.870
GENDER	179.723	1	179.723	2.265	0.167
2-Way Interations	154.614	3	51.538	0.650	0.603
SUBJECT DIET	0.045	1	0.045	0.001	0.981
SUBJECT GENDER	149.412	1	149.412	1.883	0.203
DIET GENDER	3.110	1	3.110	0.039	0.847
3-Way Interations	397.742	1	397.742	5.013	0.052
SUBJECT DIET GENDER	397.742	1	397.742	5.013	0.052
Explained	1249.532	7	178.505	2.250	0.128
Residual	714.104	9	79.345		
Total	1963.635	16	122.727		

Main subject effect; *, P<0.05 significant difference.

Table 1.5. A 3-way ANOVA to show the effects of subject, diet and gender on plasma cortisol (09:30 hrs) concentration.

Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	879.879	3	293.293	5.313	0.022*
SUBJECT	218.022	1	218.022	3.949	0.078
DIET	416.628	1	416.628	7.547	0.023*
GENDER	189.616	1	189.616	3.435	0.097
2-Way Interations	903.368	3	301.123	5.455	0.021*
SUBJECT DIET	256.150	1	256.150	4.640	0.060
SUBJECT GENDER	350.575	1	350.575	6.350	0.033*
DIET GENDER	238.856	1	238.856	4.327	0.067
3-Way Interations	86.176	1	86.176	1.561	0.243
SUBJECT DIET GENDER	86.176	1	86.176	1.561	0.243
Explained	1175.789	7	167.970	3.043	0.062
Residual	496.849	9	55.205		
Total	1672.638	16	104.540		

*, $P<0.05$ significant difference.

Table 1.6. Plasma KYN concentrations of TS subjects and healthy controls (NC) measured for 11:00hrs and 12:00hrs sample times via HPLC and the Joseph and Risby (1975) assay respectively.

	Subject status	**KYN 11:00 hours (μmol/L)**	**KYN 12:00 hours (μmol/L)**
1	NC	1.10	1.36
2	NC	1.84	1.13
3	NC	1.42	1.38
4	NC	1.70	1.58
5	NC	1.22	1.56
6	NC	1.55	1.43
Mean		1.47 ± 0.12	1.41 ± 0.07
7	TS	1.18	1.56
8	TS	1.14	1.39
9	TS	1.17	1.87
10	TS	2.14	1.80
11	TS	1.19	1.33
12	TS	1.05	1.04
13	TS	1.31	1.27
14	TS	1.30	0.92
15	TS	0.84	0.87
16	TS	1.15	1.34
17	TS	1.02	1.48
Mean		1.23 ± 0.10	1.35 ± 0.10

Table 1.7. Correlation coefficients of plasma KYN concentrations obtained from volunteers (TS subjects and controls) at various time parameters and measured by HPLC or via the Joseph and Risby (1975) method.

HPLC=sample values measured by HPLC; J & R=sample values measured by Joseph and Risby (1975) method. (Correlation analysis via Pearson product-moment coefficients).

	Correlation Coefficients			
	KYN 09:30 hrs (HPLC)	KYN 11:00 hrs (HPLC)	KYN 12:00 hrs (J & R)	KYN` 14:00 hrs (HPLC)
KYN 11:00 hrs (HPLC)	0.9535 (17) P= 0.000			
KYN 12:00 hrs (J & R)	0.3823 (17) P= 0.130	0.3598 (17) P= 0.156		
KYN 14:00 hrs (HPLC)	0.9057 (17) P= 0.000	0.9450 (17) P= 0.000	0.4047 (17) P= 0.107	
KYN 15:00 hrs (HPLC)	0.9031 (17) P= 0.000	0.8947 (17) P= 0.000	0.3302 (17) P= 0.195	0.9446 (17) P= 0.000

(Coefficient / Cases / 2-tailed significance)

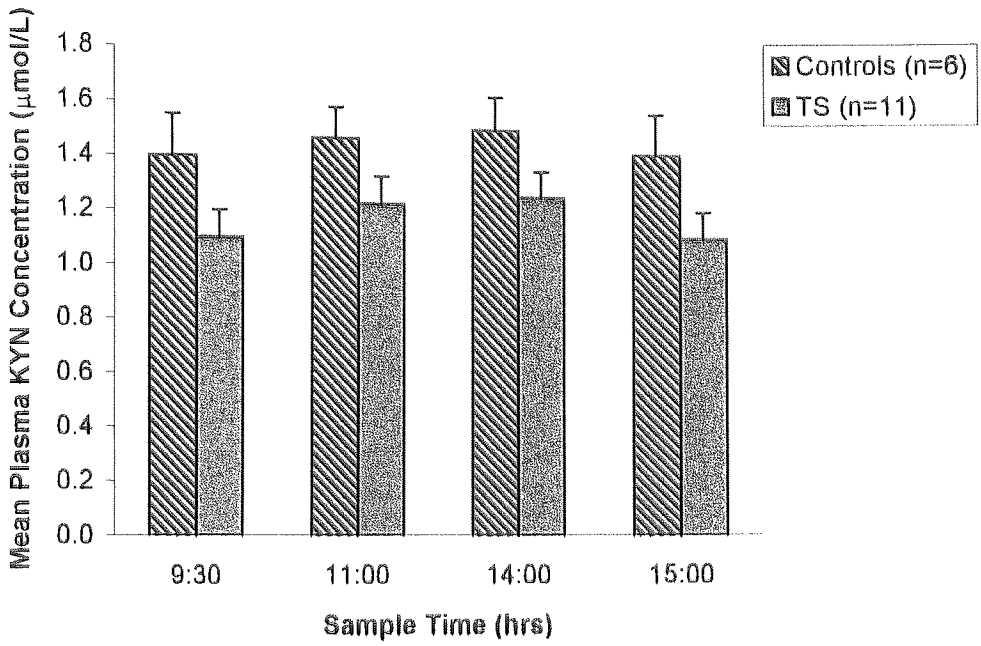

Figure 1.1. Variation of plasma KYN concentration in TS subject and control groups at different sample times.

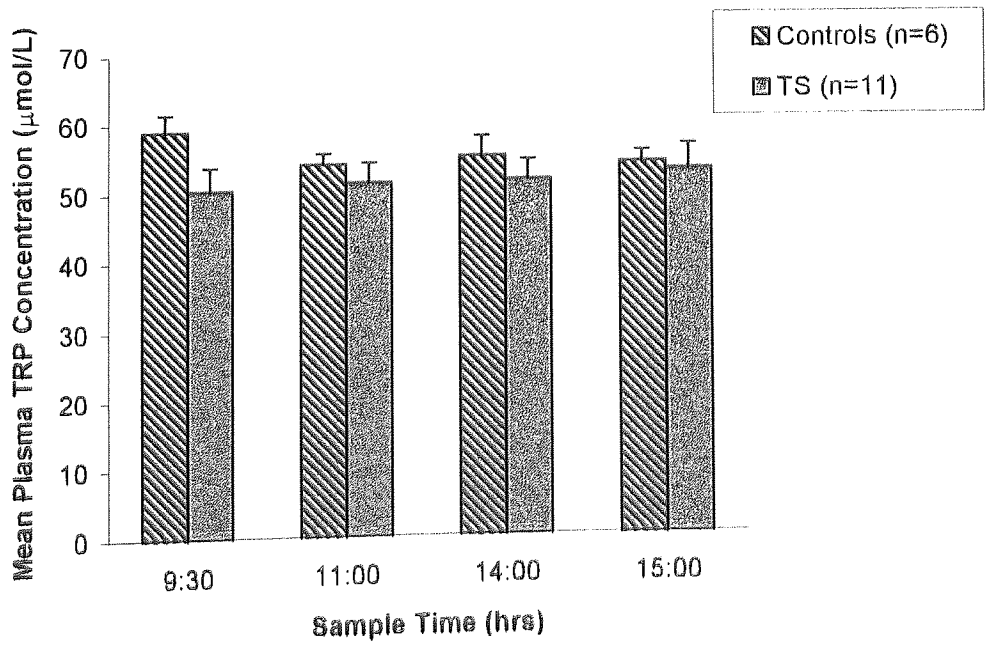

Figure 1.2. Variation of plasma TRP concentration in TS subject and control groups at different sample times.

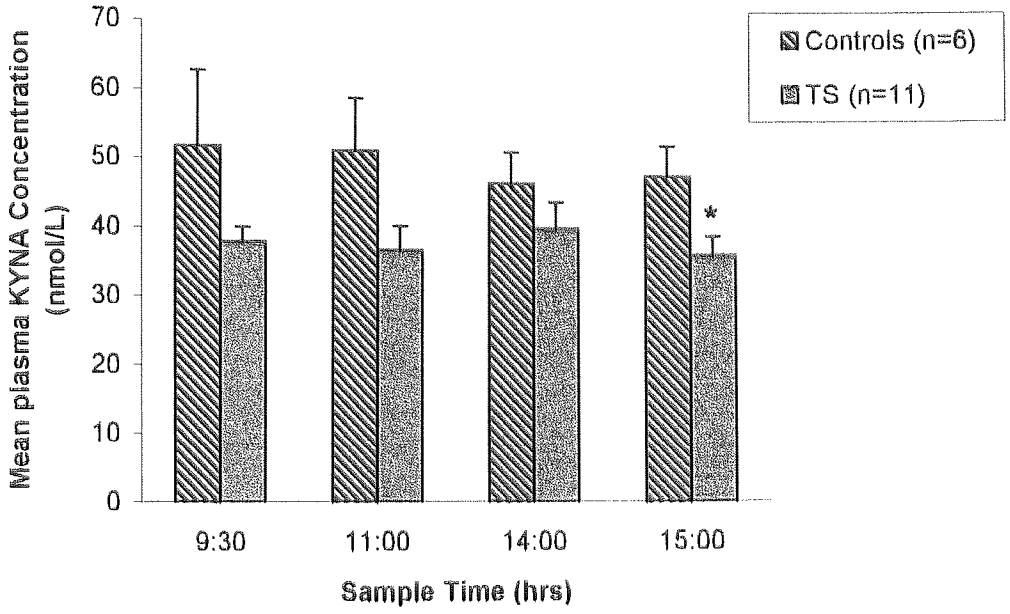

Figure 1.3. Variation of plasma KYNA concentration in TS subject and control groups at different sample times.

*, $P<0.05$ significant difference cf. controls (Student's t-test).

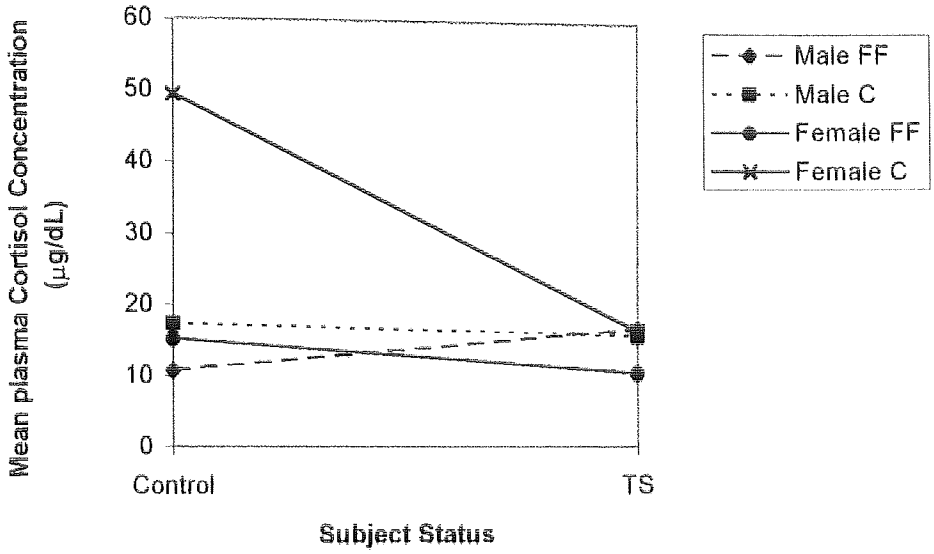

Figure 1.4. Mean cell plot for plasma cortisol concentration at the 09:30 hrs time parameter.

Sample sizes: Controls: male FF, n=2; male C, n=2; Female FF, n=1; Female C, n=1.
TS: male FF, n=5; male C, n=2; Female FF, n=1; Female C, n=3.

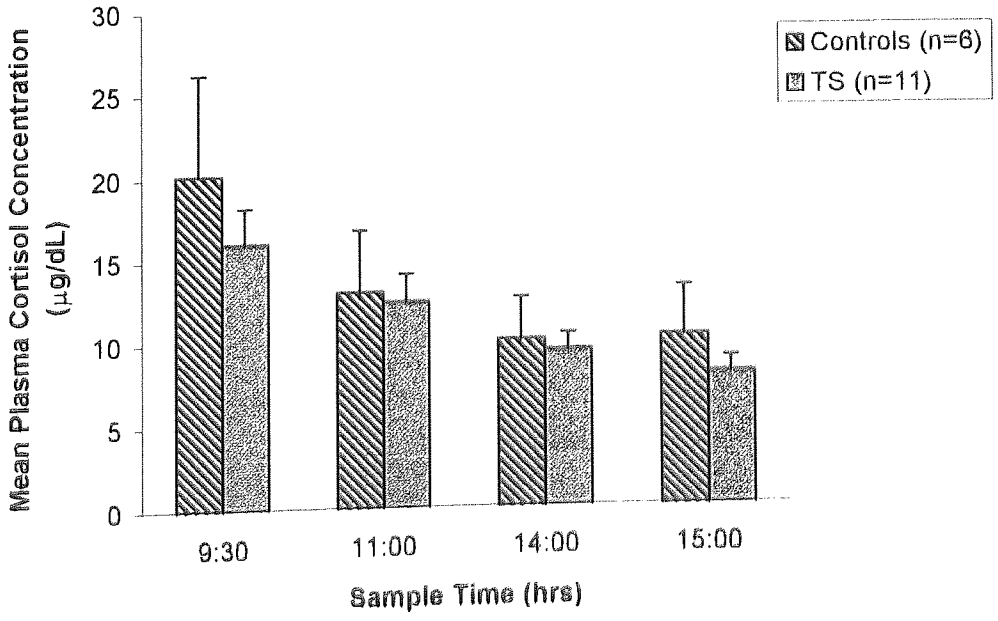

Figure 1.5. Variation of plasma cortisol concentration in TS subject and control groups at different sample times.

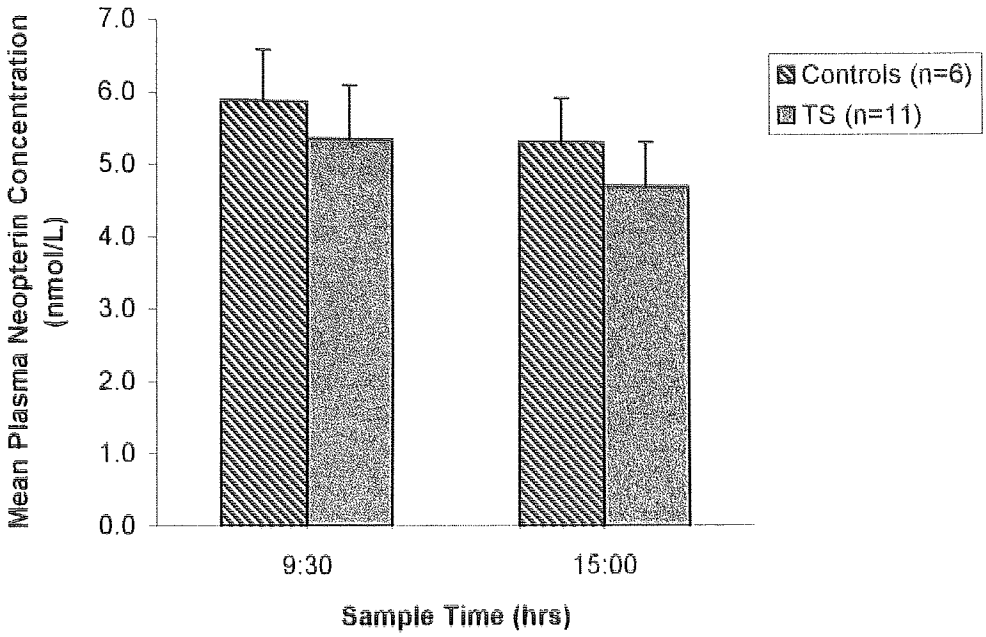

Figure 1.6. Variation of plasma neopterin concentration in TS subject and control groups at the 09:30 hrs and 15:00 hrs time parameters.

CHAPTER 2

A CROSS-SECTIONAL STUDY OF PLASMA KYNURENINE, NEOPTERIN AND RELATED SUBSTANCES IN TOURETTE'S SYNDROME.

CHAPTER 2

Introduction

Reduced plasma TRP has previously been reported in TS patients (Leckman *et al.*, 1984; Comings, 1990a). In a more recent study, no change in TRP was evident, but plasma KYN was elevated in all 7 fasting TS patients compared with 10 fasting healthy controls (Dursun *et al.*, 1994a). The mechanism of this latter change was unclear. In addition, KYN has been shown to increase tic-like behaviour in an animal model of TS (McCreary and Handley, 1995).

KYN is the first stable metabolite of the multienzyme kynurenine pathway, which acts as the major route of TRP metabolism (refer to: General Introduction). The initial and rate-limiting step of this pathway occurs via hepatic TDO or extra-hepatic IDO, which are strongly induced by cortisol and cytokines (particularly interferon-γ) respectively (see: General Introduction). Neopterin, an end-product of pteridine biosynthesis, is a commonly accepted marker of cell-mediated immunity, and is produced in and secreted by macrophages (Wachter *et al.*, 1992). Significantly, GTP-cyclohydrolase I (the first enzyme of the pteridine biosynthetic process), like IDO, is strongly induced by interferon-γ. Consequently, increased neopterin levels have been found in patients suffering from various diseases involving cellular immune activation, for example cancer, human immunodeficiency syndrome and bacterial infections of the CNS (Wachter *et al.*, 1979; Heyes *et al.*, 1992a; Heyes *et al.*, 1995).

The kynurenine pathway gives rise to several biologically active compounds, some of which have neurotoxic or neuroprotective properties, notably QUIN and KYNA (refer to: General Introduction). Alterations in the concentration of these neuroactive kynurenines has been reported in patients and animal models with inflammatory and neurologic conditions, including physical trauma to the CNS, microbial infections, various forms of meningitis and autoimmune conditions (Heyes *et al.*, 1992a; Heyes *et al.*, 1992b; Heyes *et al.*, 1995). Furthermore, KYNA and QUIN have been speculatively linked to the aetiology of certain brain diseases, such as Huntington's chorea, epilepsy and dementia (Schwarcz, 1993).

Studies indicate that the access of QUIN and KYNA to the brain from the periphery is restricted by the blood-brain barrier (Fukui *et al.*, 1991), and that anthranilic acid (rather than HKY, see: General Introduction, Figure I.2) acts as the major source of HAA and hence QUIN in the normal brain (Baran and Schwarcz, 1990). KYNA, on the other hand, is derived from KYN, which itself may be synthesised within the brain or enter the CNS from the blood, via the large neutral amino acid carrier system (Gál and Sherman, 1980). It is likely that QUIN and KYNA operate primarily as neuromodulators of excitatory amino acid receptor function in the CNS, although their precise roles have not been elucidated (refer to: General Introduction).

It has been suggested that an association exists between streptococcal infections (specifically group A β-haemolytic streptococcal infection), antineuronal antibody production and the onset or worsening of certain neuropsychiatric disorders, including TS (Kiessling *et al.*, 1993; Allen *et al.*, 1995; Tucker *et al.*, 1996). The postulated infection is thought to stimulate the production of antibodies that cross-react with basal ganglia tissues, and trigger an immunological reaction, which is manifested as a movement disorder (Kiessling *et al.*, 1993). The basis of this hypothesis centres firstly on serological evidence of recent group A β-haemolytic streptococcal infection and elevated antineuronal antibodies in affected individuals, and secondly from reports that immunomodulatory treatments have proved beneficial to TS patients, confirmed with either prior or concomitant streptococcal infection (Allen *et al.*, 1995; Tucker *et al.*, 1996; Further details in General Introduction).

The initial objective of this large cross-sectional study was to establish the increased plasma KYN in TS patients, as originally documented by Dursun *et al.*, (1994a). Given the proposed neural activities of some kynurenine pathway metabolites, it was also our aim to investigate possible changes to TRP and certain kynurenines (specifically KYNA, HKY and HAA) in plasma collected from TS subjects and normal healthy controls. Cortisol and neopterin were measured to provide an indication of alterations in the activity of TDO and IDO respectively. In addition, anti-streptolysin O (ASO) content was assessed, to ascertain whether any of the TS subjects and controls had recently contracted a streptococcal infection. A wide range of individual characteristics and environmental factors were recorded for all

candidates, and detailed assessment of the core and associated symptoms were carried out in the TS patients. It was hoped that by the wealth of information generated, important clues towards the precise biochemical background of TS (and associated conditions) might be revealed, and allow us to assess the wider implications of any changes. In the long run, this could form the foundation for the development of more effective patient management.

Additional methods

Clinical Aspects

The study consisted of 72 TS subjects and 46 controls of various ages, which included children and adults (see: Figure 2.1. for a chart of the age distributions). The mean ages of the TS subject and control groups was 21.0 ± 1.3 and 19.0 ± 1.3 years respectively. The TS group was composed of 50 male and 22 female patients, and the control group contained 25 male and 21 female volunteers. The sources of TS subjects and controls is detailed in the General Methods section.

Clinical diagnoses and psychiatric evaluations were carried out as described in the General Methods (see: Assessment tools), and were all conducted by a suitably qualified physician. Extensive details relating to demographics, clinical details and environmental stimuli were recorded too. These included age, gender, smoking status, time of year when each blood sample was collected and current use of medication for all individuals, as well as clinical characteristics, associated features and family history of tic disorders in the TS cohort. Tables 2.1 to 2.4 provide summaries of all the relevant information described. In addition, Figure 2.2 illustrates the severity range of TS cases, as evaluated via the YGTSS (Leckman *et al.*, 1989).

The exclusion criteria for the healthy, matched normal controls (NC) is contained in the General Methods section. Both TS subjects and controls were also requested to report any recent signs of cold symptoms/infections experienced around the period of blood sample donation.

Dietary status of TS subjects and controls in cross-sectional study

The TS clinic at the National Hospital (Queen Square, London) receives referrals from all around the country, and it was often necessary for subjects to travel long distances. Furthermore, the somewhat lengthy consultations and clinical assessment procedures, meant that in some cases blood samples were not collected until late afternoon (up to 17.00 hrs). Thus, after careful consideration of these ethical factors, and given the results of the pilot study (which indicated that dietary status did not have a marked effect on plasma TRP, KYN, KYNA, cortisol or neopterin levels), it was decided that subjects should not have to starve themselves until blood samples had been taken. Minimal dietary restrictions were applied, however, with all volunteers being requested to avoid intake of aspirin, nicotine and caffeine containing substances (for example cola drinks, tea, coffee and chocolate) from 0:00 hrs of the night preceding blood sample donation. These restrictions were based on evidence that these substances can alter TRP metabolism (McArthur and Dawkins, 1969; Orlikov and Ryzov, 1991; Silver and Sanberg, 1993; Dursun *et al.*, 1994a).

Subjects and controls were documented according to their dietary status as follows; 'Fasting' individuals described those who had consumed only water since midnight of the previous day. 'Restricted diet' candidates were those who had avoided intake of aspirin, nicotine and caffeine on the day of the study, and 'broken restriction' referred to any who had violated the dietary restrictions (information summarised in Table 2.5).

Measurement of Biochemical variables

Immediately after aseptic collection, the blood was placed in lithium-heparinised tubes, which were centrifuged, and the resulting plasma was collected in Eppendorf tubes, and then stored at -70°C until being assayed (Full details in General Methods). Plasma concentrations of KYN, TRP, KYNA, HKY, HAA (by HPLC methods) and cortisol (radioimmunoassay) were measured via the procedures described in the General Methods. Plasma neoptein (native) was quantified at University of Innsbruck (Austria) courtesy of Professor D. Fuchs.

Estimation of ASO content was also carried out (see: General Methods).

In addition, we had hoped to be able to provide plasma concentrations for additional TRP metabolites 5-HT and 5-HIAA, following an offer to carry out these assays by Mr. P. Whitaker (Chemical Pathology Department, Leicester Royal Infirmary). However, this was not possible due to problems of platelet contamination in the majority of plasma samples, which meant that the assay was unviable.

Blood sample collection and clinical assessments were performed over a period of 14 months, which allowed us to amass a considerable number of samples and clinical data. Biochemical variables were measured in as many of these individuals as possible, although in some cases we were hindered by the small volume of plasma which was made available to us.

Statistical Analysis

All values quoted are means ± SEM. A student's test was used to determine differences between two groups of treatment. Significance levels are two-tailed unless otherwise stated. In cases of a directional hypothesis, one-tailed probabilities were used.

Analysis of variance (ANOVA) was used for comparison of parametric data from more than two groups, followed by a *post-hoc* test where significant effects were found.

Comparison of non-parametric frequency data was performed using the Chi^2 test.

Biochemical data were also compared where appropriate using correlational analysis. The Pearson product-moment correlation coefficient was used for determining the correlation of parametric data, and the Spearman correlation coefficient was applied for data involving rating scales.

Results

Clinical rating scales in TS patient and control groups

The mean rating scores for anxiety (STAI), depression (BDI) and obsessive (LOI) behaviours were all significantly higher in the TS group (see: Table 2.1).

Plasma KYN was negatively correlated with both STAI ($r=-0.424$, $p<0.05$; $n=29$) and BDI ($r=-0.384$, $p<0.05$; $n=30$) ratings in the control sample group, although

there was no significant relationship observed in the TS category. A significant negative correlation was demonstrated between plasma TRP concentration and STAI ($r=-0.438$, $p<0.05$; $n=29$), BDI ($r=-0.493$, $p<0.01$; $n=30$) and LOI (trait + symptom scores; $r=-0.531$, $p<0.01$; $n=30$) ratings in the TS subject group only.

In the TS group, the prevalence of coprolalia was 36.8% (refer to: Table 2.2), which compares favourably with recent estimates of 33-37% from American and U.K. studies (Comings and Comings, 1985; Shapiro and Shapiro, 1986; Robertson *et al.*, 1988). Echolalia was found in 54.4% of the TS patients, which is higher than the 20-44% range compiled in a review by Robertson (1989).

Of the disorders frequently associated with TS patients, SIB occurred in 40.0% of our TS sample group compared with an estimate of 33% SIB by Robertson *et al.*, (1989), and OCB was found in 86.2% of our TS subjects.

Biochemical variables in TS patients and controls

There was no significant change in plasma TRP levels between the TS and control (NC) groups (NC=47.3 ± 1.8, TS=49.0 ± 1.0 μmol/L; see Figure 2.3). However, the TS group exhibited a significantly higher KYN concentration than the controls (NC=1.29 ± 0.06, TS=1.43 ± 0.05 μmol/L; $p<0.05$, one-tailed t-test; Figure 2.4). This was accompanied by a significant elevation in plasma neopterin within the TS subject group (NC=6.89 ± 0.31, TS=8.65 ± 0.68 nmol/L; $p<0.05$; Figure 2.5), although plasma cortisol levels (NC=14.3 ± 1.1, TS=12.4 ± 0.8 μg/dL; Figure 2.6) were unaltered.

In addition to the above differences, neopterin was significantly correlated with KYN in controls ($r=0.436$, $p<0.01$; $n=46$) and even more strongly in TS subjects ($r=0.613$, $p<0.001$; $n=71$) (shown in: Figures 2.7a and 2.7b). Cortisol, on the other hand, was not correlated with KYN in TS subjects or controls. Furthermore, there was a weak negative correlation between plasma TRP and neopterin in TS patients ($r=-0.265$, $p<0.01$; $n=71$; Figure 2.8), but no significant effect in the controls.

No significant change in HKY (NC=25.3 ± 1.4, TS=26.0 ± 1.2 nmol/L; Figure 2.9), HAA (NC=18.0 ± 1.6, TS=20.0 ± 2.5 nmol/L; Figure 2.10) or KYNA (NC=55.7 ±

4.0, TS=53.1 ± 2.1 nmol/L; Figure 2.11) concentrations were found between TS subject and control groups.

Presence of infection/cold symptoms

Within the control group there was 3 reports of recent bacterial or viral infection, and 1 report from the TS group. When these cases were excluded from the sample group, the difference in KYN between subjects and controls was slightly greater, and showed a higher level of significance than previously found (NC=1.25 ± 0.06, TS=1.44 ± 0.05 μmol/L; $p<0.02$, one-tailed t-test). However, no effects were evident for any of the other biochemical variables.

Dietary factors

The summary of dietary status (Table 2.5) shows that 12 TS patients were guilty of breaking the dietary restrictions detailed in the methods. The fasting cases (all but 2 of whom were controls), corresponded predominantly to the control individuals recruited from Birmingham Childrens Hospital (refer to: General Methods).

It was not possible to carry out a two-way ANOVA (for subject and diet effects), because there were no 'broken restriction' cases in the control group. However, a one-way ANOVA (for diet effects; analysing all cases) showed that dietary status appeared to influence plasma TRP [$F_{(2, 117)}=14.11$; $p<0.0001$]. We therefore analysed these effects in TS patient and control groups separately. In the control group (which contained no 'broken restriction' cases), plasma TRP was significantly lower in the 'fasting' (FT, n=14) volunteers compared with those classified as 'restricted diet' (RD, n=32) (FT=38.0 ± 2.1, RD=51.4 ± 2.1 μmol/L; Student's t-test, $p<0.001$). In the TS group, the 'fasting' patients exhibited significantly lower TRP than those defined as 'restricted diet' [$F_{(2,71)}=5.11$, $p<0.01$; *post hoc* Tukey's test, $p<0.05$] (see: Figure 2.12). However, since there was only 2 'fasting' TS patients, this latter effect was not considered reliable. Finally, we compared plasma TRP between TS subjects and controls in the 'restricted diet' cases only, but no difference in TRP was exhibited after applying this selection criterion (NC=51.4 ± 2.1, TS=50.1 ± 1.1 μmol/L; $p=0.59$). Therefore, although TRP appears to be influenced by diet in this study, the

small numbers of candidates within certain subgroups makes it extremely difficult to carry out a full and detailed analysis of the data.

No such dietary effects were detected in the data relating to KYN, neopterin, cortisol or the remaining biochemical variables.

Seasonal factors

It is important to acknowledge that the biochemical variables may be subject to seasonal factors, and this appears to be most clearly demonstrated by the change in cortisol with time of year (see: Figure 2.13). A marked seasonal variation in cortisol has previously been reported (Handley *et al.*, 1980), and we cannot discount the possible effects of these fluctuations on the activity of the kynurenine pathway in this study. Indeed, although our TS subject plasma samples were collected gradually throughout the course of the year, the vast majority of the controls were obtained during the month of March (see: Table 2.2).

In an effort to take these seasonal factors into account, we tried analysing only those samples obtained during the months March to June, which was intended to represent a time of year less exposed to the extremes of the Summer and Winter temperatures, and which also included virtually all of the control plasma samples (n=44) and a reasonable sample size from those donated by the TS cohort (n=27). This approach indicated that the differences in KYN (NC=1.28 ± 0.07, TS=1.53 ± 0.08 μmol/L; $p<0.02$, one-tailed t-test) and neopterin (NC=6.92 ± 0.32, TS=8.81 ± 0.66 nmol/L; $p<0.01$) for this period were greater (see: Figures 2.14 and 2.15), and statistically more significant, than the data for the total length of the study. The TS subjects from this sample also exhibited a closer correlation between KYN and neopterin ($r=0.718$, $p<0.001$; n=27) compared with the entire TS group.

Smoking status

TS subjects who smoked (TS_S) exhibited significantly lower plasma KYN concentrations than non-smoking TS subjects (TS_{NS}) (TS_S=1.15 ± 0.10, TS_{NS}= 1.48 ± 0.05 μmol/L; $p<0.05$; Figure 2.16). Unfortunately, comparisons based on smoking

status could not be made for the controls, since there was only two smokers within this group.

A reduction in plasma KYNA was also found in TS smokers compared with their non-smoking counterparts (TS_S=40.6 ± 3.3, TS_{NS}=55.0 ± 2.3 nmol/L; $p<0.05$; Figure 2.17). Although a similar trend towards a decrease in the levels of neopterin and the other KYN pathway metabolites was also found in TS smokers, the differences were not significant.

In light of these findings and reports that nicotine may ameliorate TS symptoms, we examined whether the YGTSS may differ according to smoking status, but no difference was detected (YGTSS: TS_S=31.9 ± 3.2, n=8; TS_{NS}=27.5 ± 1.3, n=61; p=0.27) .

There was a slightly closer correlation between neopterin and KYN in the non-smoking TS subjects ($r=0.646$, $p<0.001$; n=61) than in the subject group as a whole.

Age effects

Statistical analyses indicated a significant correlation between TS subject age and plasma cortisol concentration (Spearman correlation coefficient, $r=-0.430$; $p<0.001$; n=69), although this relationship was not reflected in the controls. No further relationships were demonstrated between age of subject and the biochemical variables.

A negative correlation between subject age and ADHD rating (DSMIV) was found (Spearman correlation coefficient, $r=-0.729$, $p<0.001$; n=24; Figure 2.18). There was also a weak positive correlation between age of individual and the LOI score for all cases scored (Spearman correlation coefficient, $r=0.279$; $p<0.05$; n=63), but this effect was not significant if TS subject and control groups were analyed separately.

Gender Effects

The proportion of males in the control group (54%) should ideally have been more closely matched to the TS subject group (69%), to reflect the mean sex ratio of the condition, which is estimated to be between three and four times more common in males than in females (see: General Introduction). Nevertheless, the high sample

numbers ensured that appropriate analyses could be performed to assess gender effects.

There was a trend towards higher YGTSS in male TS subjects (n=45) than in females (n=19) (YGTSS; males=29.5 ± 1.5, females=24.1 ± 2.5; p=0.054).

A two factor ANOVA (as a function of subject and gender) indicated a highly significant gender effect on the plasma KYNA concentration [$F_{(1, 114)}$=8.54, p<0.01] (see: Table 2.6). Using both TS subject and control cases, males (n=74) showed significantly higher plasma KYNA than females (n=41) (KYNA; male=58.4 ± 2.6, female=47.1 ± 3.2 nmoles/L; p<0.01). Analysis of control samples only, indicated that male controls (NC_{male}, n=25) had significantly higher KYNA than female controls, (NC_{female}, n=20) (NC_{male}=63.5 ± 5.6, NC_{female}=47.2 ± 5.5 nmoles/L; p<0.05). Although a similar trend was observed in the TS males and females (TS_{male}, n=49; TS_{female}, n=21), the difference was not significant. (TS_{male}=55.8 ± 2.6, TS_{female}=47.1 ± 3.5 nmoles/L; p=0.064).

No other gender influences were found.

Family History of Tics

The widely accepted heriditary nature of TS, meant that a high proportion of TS subjects testified to having some history of tics in their family. Significantly lower plasma TRP levels were found in TS patients reporting a family history of tics (TS_{FH}, n=55) compared with subjects with no known history of tics in their family ($TS_{no\ FH}$, n=10) (TRP; TS_{FH} = 47.1 ± 1.1, $TS_{no\ FH}$ = 58.1 ± 2.9, μmol/L; p<0.0001; see Figure 2.19). In addition, although plasma KYN between these two subgroups was unchanged, the KYN:TRP ratio was significantly elevated in TS patients with a family history of tics (KYN:TRP; TS_{FH} = 0.031, $TS_{no\ FH}$ = 0.023, p<0.05). Hence, there may in fact be a higher turnover of TRP in those TS patients, where there is an established genetic background of the disorder. No difference in neopterin, cortisol or the remaining biochemical variables was found on this basis.

ASO titres

There was no evidence to indicate that there was any significant difference in the frequency of positive ASO titres between subjects and controls (Chi^2=2.36, p=0.500), with very little difference between the expected and observed frequencies (see: Table 2.7).

Since the frequency of positive ASO titres (200 IU/ml) may vary according to age, and because TS is a condition which first appears in childhood, we investigated how the frequency of positive readings might be affected by the age of our subjects and controls. Selection of cases exclusively below the age of 16 years did not demonstrate any change in the number of positive titres between subjects and controls (Chi^2=0.865, p=0.352; Table 2.8). Crosstabulation statistics for our subjects and controls, when divided into different age groups, did not reveal any significant effects either (Tables 2.9 and 2.10), although the 0 to 10 year age range in the subject group cell showed a slightly elevated number of positive ASO readings compared with the expected frequency.

Current medication

Kynurenine pathway variables of unmedicated control (n=21) and TS subject (n=14) groups were initially compared, but no differences were observed between the two groups (KYN, TS=1.45 ± 0.08, NC=1.35 ± 0.08 μmol/L, p=0.378; TRP, TS=53.2 ± 2.5, NC=46.7 ± 3.2 μmol/L, p=0.125; Neopterin, TS=7.95 ± 0.53, NC=7.34 ± 0.43 nmol/L, p=0.377; Cortisol, TS=11.7 ± 1.3, NC=15.1 ± 2.0 μg/dL, p=0.229). Nonetheless, in agreement with our original hypothesis, KYN (as well as neopterin) was higher in the TS cases.

The wide range of medications received by many of the TS subjects (as well as the controls) for various conditions, including TS and its associated disorders, but also for depression, anxiety, epilepsy and asthma, was the main obstacle to assessing specific drug effects on the biochemical variables. In addition, few of our TS subjects were receiving identical drug treatments, which further hindered statistical comparisons between these sample groups. However, comparison of the biochemical variables in unmedicated TS patients (n=14) and groups of similarly medicated TS

patients, more specifically with the drugs haloperidol (n=12), sulpiride (n=9) and clonidine (n=7) were possible.

No changes were observed between umedicated TS (TS_{Unmed}) patients and those treated with either haloperidol or sulpiride, but differences were exhibited when comparing unmedicated TS subjects with TS patients taking clonidine (TS_{Clon}). The clonidine group showed a trend towards reduced plasma KYN (TS_{Unmed}=1.45 ± 0.08, TS_{Clon}=1.19 ± 0.11 μmol/L, p=0.084; see Figure 2.20), as well as a significant decrease in TRP (TS_{Unmed}=53.2 ± 2.5, TS_{Clon}=43.7 ± 2.4 μmol/L, p<0.05; see Figure 2.21) and KYNA (TS_{Unmed}=53.0 ± 4.4, TS_{Clon}=39.8 ± 2.4 nmol/L, p<0.05; see Figure 2.22). There is no reason to expect clonidine alone to affect the plasma concentrations of these biochemical variables. Nonetheless, replication of these differences in clonidine treated TS subjects both before and after a course of medication would offer greater credence to this finding.

Associated features

Differences in the biochemical variables were investigated in the TS patients with comorbid disorders. The task was complicated in subjects with ADHD, since assessment of patients recruited earlier in the study was based on DSMIII-R (A.P.A., 1987) criteria, while DSMIV (A.P.A., 1995) was used to score patients from the latter part of study. This discrepancy was both unfortunate and unintended.

Nevertheless some interesting relationships came to light, for example there was a negative correlation between ADHD score (using DSMIV) and plasma KYN concentration (Spearman correlation coefficient, r=–0.439, p<0.05; n=23) in affected subjects (see: Figure 2.23). A strong correlation was also found between ADHD rating (DSMIV) and plasma cortisol (Spearman correlation coefficient, r=–0.647, p<0.001; n=23) in our TS cohort (Figure 2.24).

None of the kynurenine pathway variables were significantly affected by the presence or absence of OCB in TS subjects.

Discussion

The data conforms with the hypothesis of elevated plasma KYN in TS patients, thus suggesting an abnormality of KYN metabolism in TS. Plasma neopterin, which correlated positively with KYN in both TS subjects and controls, was similarly increased in the TS group, although cortisol levels were unchanged. This implies that the source of the raised KYN emanates from the induction of IDO (rather than an alteration of TDO activity), possibly indicating an autoimmune basis for the condition. Whilst this change in neopterin seems to contradict the negative findings of Dursun *et al.* (1994a), the considerably greater number of TS subject and control samples collected in our study may be significant. Equally, differences in the assay procedures employed for neopterin determination may be important. For example, Dursun and colleagues used HPLC to measure neopterin, but the plasma samples in this study were quantified by means of radioimmunoassay in a laboratory where this assay is routinely performed (see: General Methods).

No difference in the plasma concentrations of HKY, HAA or KYNA was found between the control and TS group, although the passage of these substances between brain and periphery is more restricted (Fukui *et al.*, 1980) compared with TRP and KYN (Gál and Sherman, 1980; Fernstrom, 1983). Furthermore, studies have shown that the activity of IDO does vary markedly in different brain areas, with the subcortex and pineal gland being associated with high levels of enzyme activity (Gal and Sherman, 1980; Fujiwara *et al.*, 1978). Hence, it is possible that an increased flux through the kynurenine pathway may occur only in localised areas within the CNS, making any changes difficult to pick up, particularly when assays are conducted only on biological fluids of a peripheral origin. It was unfortunate that we could not quantify QUIN (refer to: General Methods), bearing in mind its well documented potential as a neuromodulator at excitatory amino acid receptors.

The weak negative correlation between anxiety score (STAI) and plasma KYN concentration in the control sample is in direct contrast to the findings of Orlikov and Ryzov (1991), where KYN was positively correlated with anxiety rating. Orlikov and Ryzov also found that at the peak of caffeine-induced anxiety, there was no relationship between anxiety indices and KYN concentration. In view of the higher anxiety scores shown by the TS subjects, this latter finding by Orlikov and Ryzov

could explain the lack of correlation between plasma KYN and anxiety within the TS cohort. In addition, the high prevalence of various psychopathologies and behavioural disorders in TS patients (see: General Introduction) correlates well with the significantly higher scores for anxiety, depression and obsessive behaviours (Table 2.1) compared with the control candidates.

In contrast to the much smaller pilot study (Chapter 1), a diet effect on plasma TRP was observed in this investigation. However, we were prevented from performing extensive statistical comparisons, due to the lack of cases in certain subject categories. If we refer to Table 2.5, we can see that of the 12 candidates who broke one or more of the restrictions laid down in the protocol, all were TS patients. This could be due to the fact that it was quite late in the day when blood samples were obtained from many of the TS subjects (see: Additional methods), and consequently they either forgot about or ignored the restrictions on nicotine and/or caffeine products. Alternatively, this could be an indication that people with TS were more impulsive, and therefore less able to resist these urges.

With reference to diurnal influences and their possible effects on the biochemical variables, it is noteworthy that the majority of the blood samples were collected late in the afternoon, a time when cortisol levels are generally more stable and not subject to their early morning peaks (see: Chapter 1). Nonetheless, we only had a limited control over the precise time of venesection in the study. In many cases, the patients had travelled considerable distances to attend the clinic, and their initial objective on arrival, would to meet the physician, in order to discuss their condition. Following the consultation, patient consent for blood sampling would be easier to secure, although the time of sample collection is undoubtedly something which deserves a great deal of thought, in view of its capacity to alter the entire biochemical picture. For example, previously documented reductions in plasma TRP in TS patients were measured in early morning samples (Leckman *et al.*, 1984; Comings, 1990), so that modifications of future protocols could enable these findings to be replicated. On the other hand, since cortisol levels in the afternoon appear to be less variable, afternoon sample times may be necessary in order to highlight the changes to KYN and neopterin in TS.

The seasonal variation of certain parameters, notably cortisol, is another factor of real significance to this type of study. Handley *et al.* (1980) reported that cortisol was influenced by time of year, and our data appears to support this phenomenon (see: Figure 2.13). Moreover, whilst the samples from the TS patients were accumulated gradually throughout the length of the study, the control samples were largely collected during the month of March, which our graph suggests is a period when cortisol levels are less susceptible to the seasonal peaks and troughs. These seasonal factors may account for our failure to detect a change in KYN in the smaller pilot study (Chapter 1), since all the plasma samples were obtained solely during the Summer months of July and August, when cortisol levels could be depressed. This theory was supported by the data for the months March to June only, when a greater elevation in KYN (see: Figure 2.14) was observed in the TS group. The level of statistical significance of the neopterin elevation in TS was similarly increased over this period (see: Figure 2.15), but although this is unlikely to be related to fluctuations in cortisol, it may indicate that neopterin itself is susceptible to seasonal influences. One could, for example, speculate that increased neopterin in TS during the Winter months might be less prominent, because of an increase in the overall number of candidates (*i.e.* controls and TS) suffering from common cold related symptoms. To recognise the importance of this factor, all volunteers for the study were requested to make any signs of infection known at the time of venesection. Consequently, we found that exclusion of the handful of cases who reported an infection, increased the level of statistical significance for raised plasma KYN in TS.

The reduction in plasma KYN (and KYNA) exhibited by TS smokers was a most interesting, if somewhat unexpected result. The potential importance of the nicotinic cholinergic system in tic disorders has previously been demonstrated, where non-smoking TS patients inadequately controlled by neuroleptics, exhibited an amelioration of tics following application of transdermal nicotine patches (Silver and Sanberg, 1993; Dursun *et al.*, 1994b). In these studies, the extent and speed of tic symptom improvement provided by nicotine, as well as the period of any therapeutic effect conveyed to patients following nicotine patch removal, varied considerably between individuals (Dursun *et al.*, 1994b; Silver *et al.*, 1996). Since the investigation

of nicotine as a potential adjunct to TS treatment is still relatively new, many questions remain answered, such as the likely mechanism of its action in tic conditions and the longer-term effects of nicotine therapy (including the possibility of nicotine addiction from chronic treatment). Further analysis of the data did not indicate a difference in the YGTSS between smokers and non-smokers. However, it could be argued that the smokers may initially have had more severe TS symptoms, and thus were effectively self-medicating with nicotine via their cigarettes. Alternatively, prolonged exposure to nicotine from smoking could lead to tolerance to its beneficial effects. Either way, it is not possible to make direct comparisons between the biochemical data of this cross-sectional investigation and the clinical studies of nicotine's potential benefits in TS. However, there is obviously scope for exploring how nicotine and the cholinergic pathway may influence tic behaviours and/or interact with the kynurenine pathway.

Although cortisol exhibited a negative correlation with age in the TS subject group, no further age-related effects were found among the other biochemical variables. In contrast, a significant increase in neopterin with age was reported in a large population of healthy adults (Diamondstone *et al.*, 1994). In the same study, mean neopterin levels were significantly lower among smokers than non-smokers. Our data failed to support either of these findings, with no apparent difference in plasma neopterin between TS smokers and non-smokers. Unfortunately, since there was only two smokers in our control group, it was not feasible to analyse the control group on this basis.

A highly significant negative correlation between age and ADHD rating was evident in the TS patients, although this relationship was not altogether surprising, bearing in mind that ADHD is a condition more commonly associated with children. We might have expected a similar association between age and YGTSS, since it has been suggested that adulthood frequently marks a time for improvement in the number and severity of tic symptoms (Cohen and Leckman, 1994). However, the natural course of the disorder is difficult to predict, and it could be construed that only a minority of mildly affected TS adults volunteered to take part in the study.

It has been widely reported that TS occurs three to four times more frequently in males than females (Corbett *et al.*, 1969; Moldofsky *et al.*, 1974; Golden, 1984; Burd *et al.*, 1986; Regeur *et al.*, 1986; Robertson *et al.*, 1988), but there appears to be little reference to differences in the biochemistry or symptoms between the two genders. The trend towards higher mean YGTSS in TS males was interesting, although there was no suggestion that the activity of the kynurenine pathway differed significantly according to the gender status of our TS sample population.

Despite the well established hereditary nature of TS, its precise genetic background is still some way from being fully understood. Numerous family studies have been presented in literature to suggest that relatives of probands with TS also exhibit the disorder or have either motor or vocal tics. Based on the figures estimated in several reports, Robertson *et al.* (1988) suggested that the prevalence of a previous family history of TS or some other tic condition in TS probands was between 12 and 67%. Of the TS patients in our study, from whom this information was obtained, the prevalence was 86% (see: Table 2.4). In those patients reporting a family history of TS or tics, significantly lower plasma TRP was found, together with an increase in the KYN:TRP ratio, which suggests that there may be a higher turnover of TRP in these individuals. No indication as to the source of this increased turnover was provided by the neopterin or cortisol data.

The results from the ASO titre tests at first glance fail to support a link between prior infection with Group A β-haemolytic streptococcal infection and the onset (or sudden worsening of symtoms) of TS and other tic disorders, as proposed by other research groups (Kiessling *et al.*, 1993; Allen *et al.*, 1995; Tucker *et al.*, 1996). However, there is an important factor which could be crucial to our lack of meaningful data. TS is a disorder which is manifested in childhood, and according to the diagnositic criteria (DSMIV) has an age of onset before 18 years (A.P.A., 1995). Indeed the mean age of onset for TS symptoms is 7 years (Robertson, 1989), and it has been estimated that symptoms have appeared in 96% of patients by the age of 11 years old (Robertson, 1989). It is thus significant that in such studies where a relationship between group A β-haemolytic streptococci and TS (or other tic disorders) was found, subjects were exclusively aged up to 14 years old (Kiessling *et al.*, 1993; Allen

et al., 1995; Tucker *et al.*, 1996). In contrast, the TS subjects within our cross-sectional investigation were not selected on the basis of either recently being diagnosed with TS, or having experienced a sudden exacerbation in their symptoms. We could therefore scarcely expect to obtain data which agreed with this hypothesis. It is noteworthy too, that Swedo and colleagues (1994) acknowledge that the theory of antineuronal antibody-mediated neuropsychiatric dysfuntion can only account for a subgroup of children with TS (or tics or OCD), given that the disorders do not always follow an episodic course, have an abrupt onset or produce increased antibodies in all subjects.

In summary, the results from this large cross-sectional study appear to confirm that plasma KYN is increased in TS patients. However, the increase is reflected as a general elevation in KYN within the TS group, rather than being raised in all affected cases as originally documented by Dursun *et al.* (1994a). The higher plasma neopterin within the TS sample group suggests that an induction of IDO causes the KYN increase, although there is no indication of the precise pharmacological mechanism responsible for expression of individual TS symptoms or associated features. The development of assays to measure other kynurenine metabolites in biological fluids, particularly QUIN, can only enhance our understanding of the underlying biochemical basis of TS.

Given the promising data from various studies relating to nicotine's beneficial effects in TS, the differences in KYN and KYNA between TS smokers and non-smokers perhaps provided the greatest additional source of interest, and highlighted the potential importance of nicotinic cholinergic pathways to tic disorders. Any further studies, however, should be designed to include similar numbers of smokers and non-smokers within TS and control groups. Additional consideration toward diurnal and seasonal effects is also warranted, to ensure that any biochemical differences in TS do not pass undetected.

Table 2.1. Age, gender and mean score results of the standardised rating scales for TS patients and controls (for all cases where data was obtained).

	Controls	**TS**
Mean age (years)	19.0 ± 1.3	21.0 ± 1.3
Male : Female ratio	25 : 21	50 : 22
Smoking : Non-smoking ratio	2 : 43	10 : 61
Spielberger Trait Anxiety Index (STAI)	34.6 ± 1.8 (n=29)	49.2 ± 2.6** (n=22)
Beck Depression Inventory (BDI)	4.6 ± 0.6 (n=30)	11.9 ± 1.5** (n=30)
Leyton Obsessional Inventory (LOI)		
Symptom	N/R	15.5 ± 2.1 (n=21)
Trait	N/R	13.5 ± 1.7 (n=21)
Total (trait + symptoms)	16.8 ± 1.8 (n=30)	29.7 ± 2.6** (n=31)

**, $P<0.001$ significant difference cf. controls (Student's t-test); N/R = not reported.

Table 2.2. Summary of the number of blood samples collected from TS patients and controls during each particular month.

Month	Controls	TS Patients
July	1	3
August	0	1
September	0	2
October	0	10
November	0	2
December	0	17
January	0	4
February	1	5
March	30	9
April	5	7
May	7	5
June	2	6

Table 2.3. Details of medications at time of blood sampling in TS patients and controls.

Medications	Controls	TS Patients
Haloperidol	1	12
Pimozide	0	4
Sulpiride	1	9
Clonidine	1	7
Nicotine	0	2
Antidepressant	3	9
Hypnotic	1	2
Antimovement	1	4
Anticonvulsant	0	3
Asthma	8	5
Other	7	5

Table 2.4. Summary of clinical characteristics and prevalence of associated features in TS subjects.

Mean YGTSS	27.9± 1.3 (n=64)
Mean number or motor tics	36.0 ± 1.4 (n=65)
Mean number of vocal tics	11.1 ± 0.9 (n=65)
Coprolalia	36.8% (n=68)
Echolalia	54.4% (n=68)
Family history of tics	84.6% (n=65)
Family history of OCD	66.6% (n=66)
OCB	86.2% (n=65)
SIB	40.0% (n=68)

Table 2.5. Summary of dietary status reported by TS subjects and controls on day of blood sample collection.

	Controls	**TS Subjects**
Fasting	14	2
Restricted Diet	32	58
Broken Restrictions	0	12

Table 2.6 A 2-way ANOVA to show the effects of subject and gender on plasma KYNA concentrations

Source of Variation	Sum of Squares	DF	Mean Square	F	Significance of F
Main Effects	4137.253	2	2068.627	4.453	0.014
SUBJECT	391.790	1	391.790	0.843	0.360
GENDER	3967.979	1	3967.979	8.541	0.004**
2-Way Interations	370.377	1	370.377	0.797	0.374
SUBJECT GENDER	370.377	1	370.377	0.797	0.374
Explained	4343.905	3	1447.968	3.117	0.029
Residual	51567.726	111	464.574		
Total	55911.631	114	490.453		

Main gender effect; **, $P<0.01$ significant difference.

Table 2.7. Observed and expected values for positive and negative ASO titres in TS patients and controls at the dilution range 200 to 400 IU/ml.

Note: 0 represents negative reading.
For each cell: Top figure = observed count, Bottom figure = expected value.

ASO titre dilution (IU/ml)	Controls	TS Subjects	Row (% Total)
0	31 (32.5)	51 (49.5)	82 (70.7%)
>200	7 (5.2)	6 (7.8)	13 (11.2%)
>300	5 (4.0)	5 (6.0)	10 (8.6%)
>400	3 (4.4)	8 (6.6)	11 9.5%
Column (% Total)	46 (39.7%)	70 (60.3%)	116 (100.0%)

Chi-Square	Value	DF	Significance
Pearson	2.363	3	0.500

Table 2.8. Observed and expected values for positive (>200 IU/ml) and negative ASO titres in TS patients and controls below the age of 16 years.

For each cell: Top figure = observed count, Bottom figure = expected value.

ASO titre reading	Controls	TS Subjects	Row (% Total)
negative	12 (10.7)	20 (21.3)	32 (71.1%)
positive (>200 IU/ml)	3 (4.3)	10 (8.7)	13 (28.9%)
Column (% Total)	15 (33.3%)	30 (66.7%)	45 (100.0%)

Chi-Square	Value	DF	Significance
Pearson	0.865	1	0.352

Table 2.9. Observed and expected values for positive (>200 iu/ml) and negative ASO titres for the TS subject group over various age ranges.

For each cell: Top figure = observed count, Bottom figure = expected value.

Age Range (yrs)	Negative ASO titre 0	Positive ASO titre 1	Row (% Total)
0-10	5 (7.2)	5 (2.8)	10 (14.7%)
11-20	19 (18.0)	6 (7.0)	25 (36.8%)
21-30	15 (15.1)	6 (5.9)	21 (30.9%)
31-40	7 (5.0)	0 (2.0)	7 (10.3%)
>40	3 (3.6)	2 (1.4)	5 (7.4%)
Column (% Total)	49 (72.1%)	19 (27.9%)	68 (100.0%)

Chi-Square	Value	DF	Significance
Pearson	5.689	4	0.224

Table 2.10. Observed and expected values for positive (>200 IU/ml) and negative ASO titres for the control group over various age ranges.

For each cell: Top figure = observed count, Bottom figure = expected value.

Age Range (yrs)	Negative ASO titre 0	Positive ASO titre 1	Row (% Total)
0-10	5 (4.2)	1 (1.8)	6 (13.6%)
11-20	9 (9.9)	5 (4.1)	14 (31.8%)
21-30	15 (14.8)	6 (6.2)	21 (47.7%)
31-40	1 (1.4)	1 (0.6)	2 (4.5%)
>40	1 (0.7)	0 (0.3)	1 (2.3%)
Column (%Total)	31 (70.5%)	13 (29.5%)	44 (100.0%)

Chi-Square	Value	DF	Significance
Pearson	1.565	4	0.815

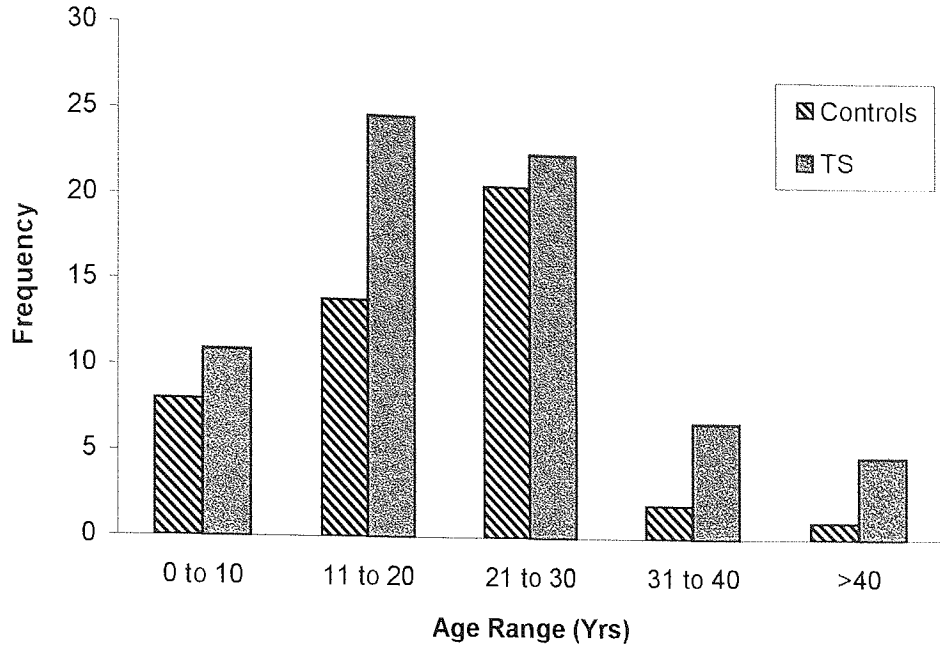

Figure 2.1. Chart to represent the age range distribution in TS subject and control groups of the cross-sectional study.

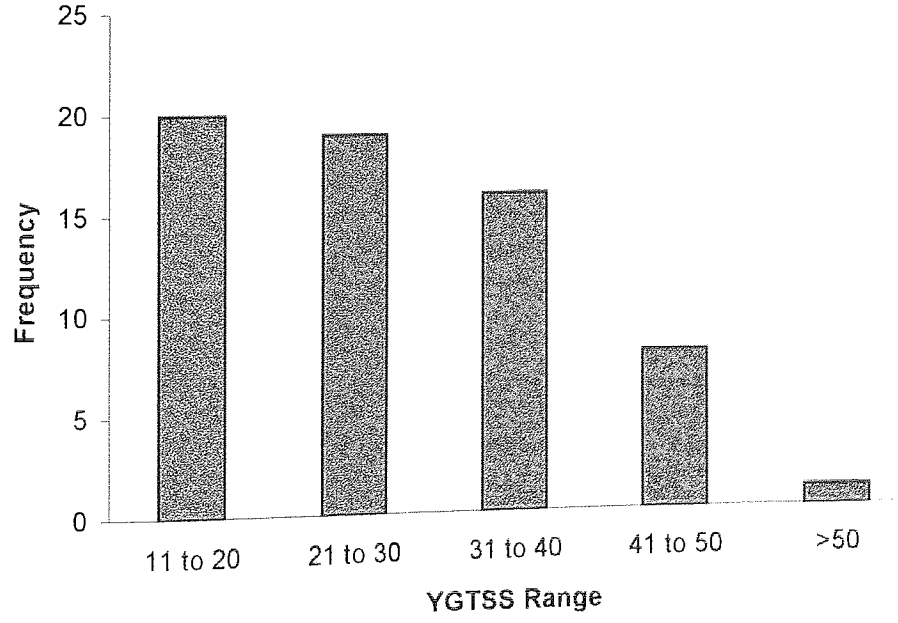

Figure 2.2. Chart to show the frequency of the YGTSS range recorded for the TS subject group of the cross-sectional study.

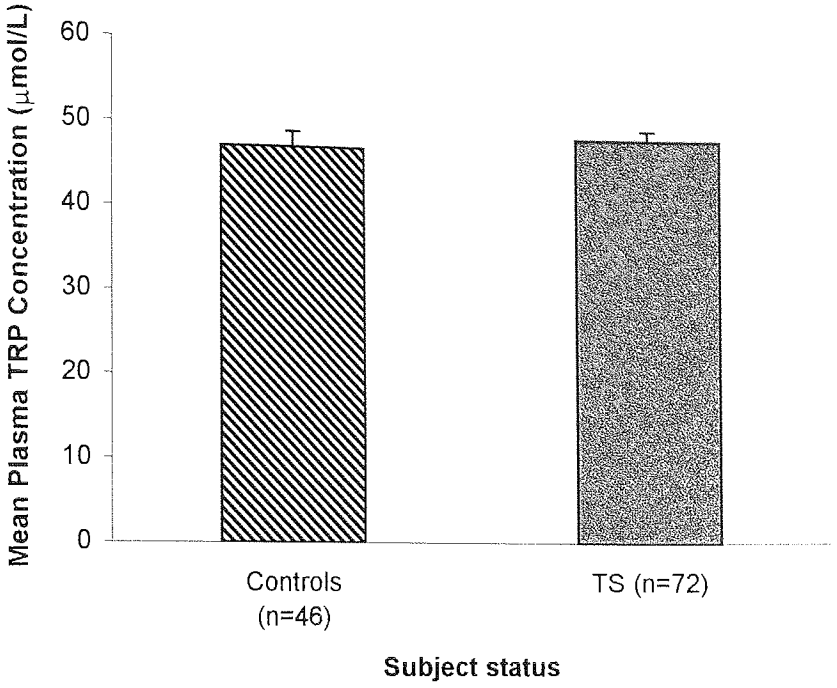

Figure 2.3. Plasma TRP concentrations in TS subject and control groups.

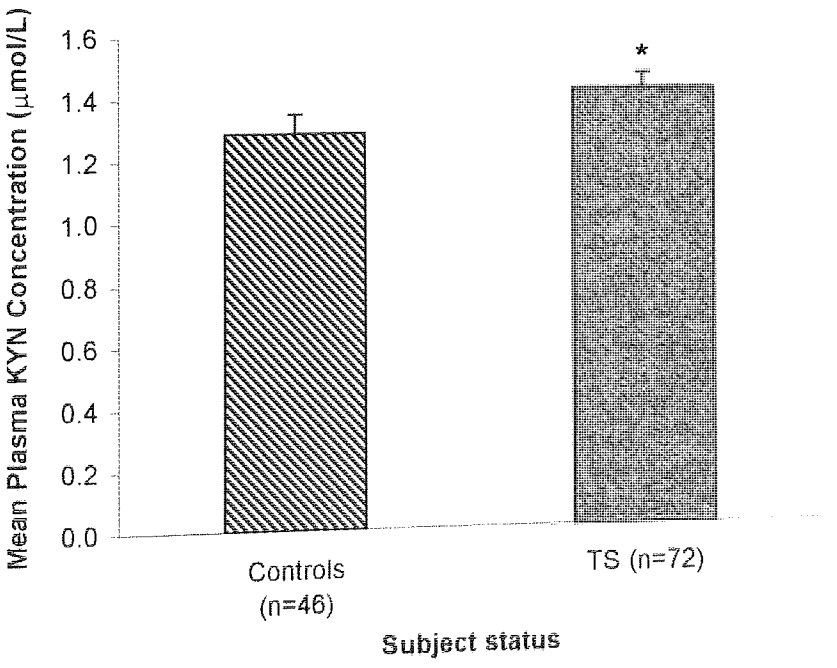

Figure 2.4. Plasma KYN concentrations in TS subject and control groups.

*, $p<0.05$ significant difference cf. controls (Student's t-test; one-tailed).

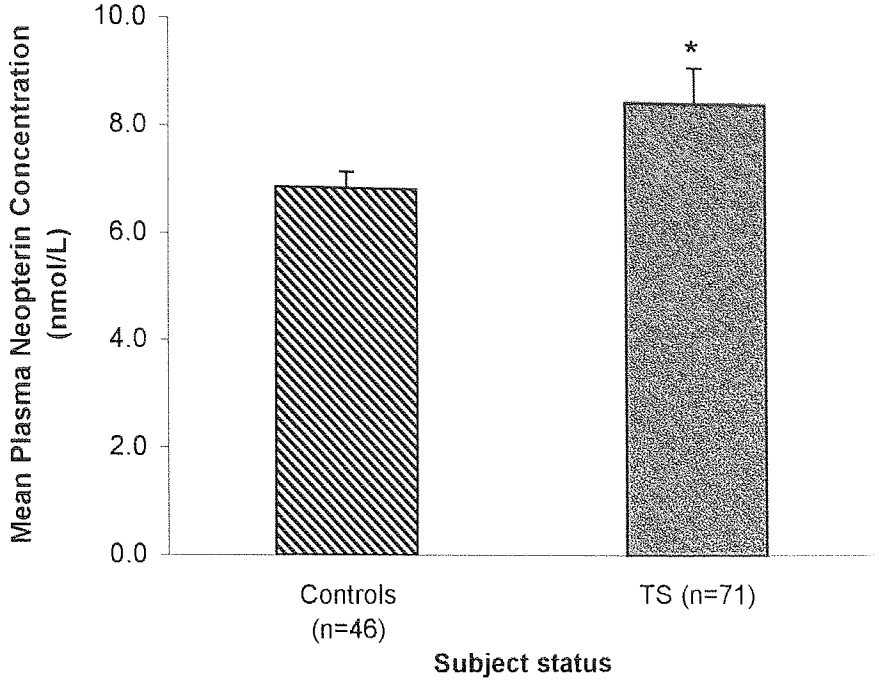

Figure 2.5. Plasma neopterin concentrations in TS subject and control groups.

*, $p<0.05$ significant difference cf. controls (Student's t-test).

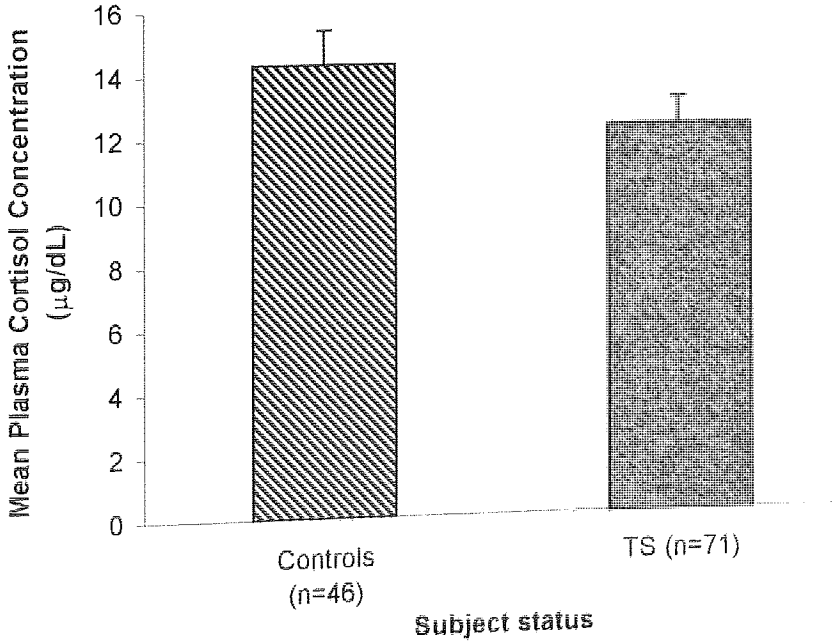

Figure 2.6. Plasma cortisol concentrations in TS subject and control groups.

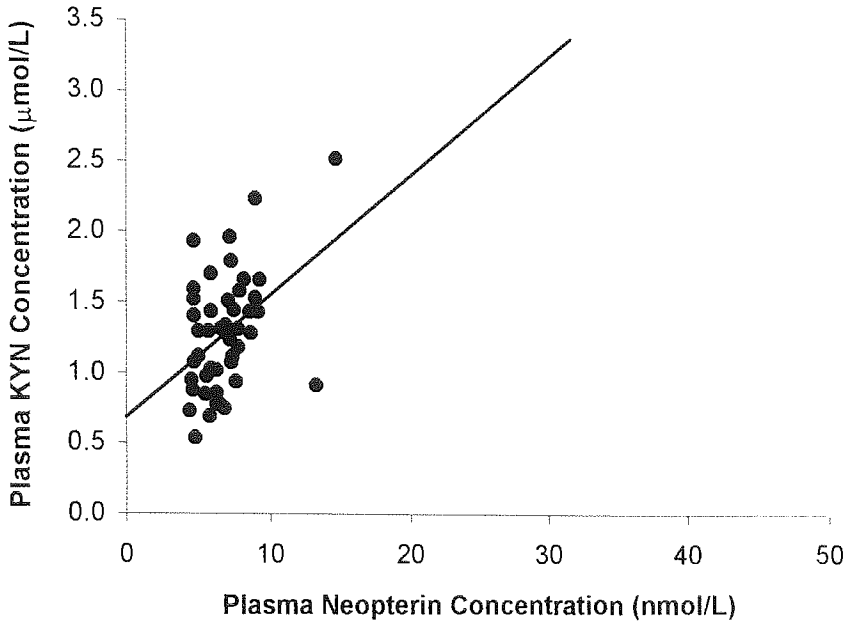

Figure 2.7a. Correlation between plasma KYN and neopterin concentrations in the control group.

(r=0.436; p<0.01, Pearson product-moment correlation coefficient; n=46).

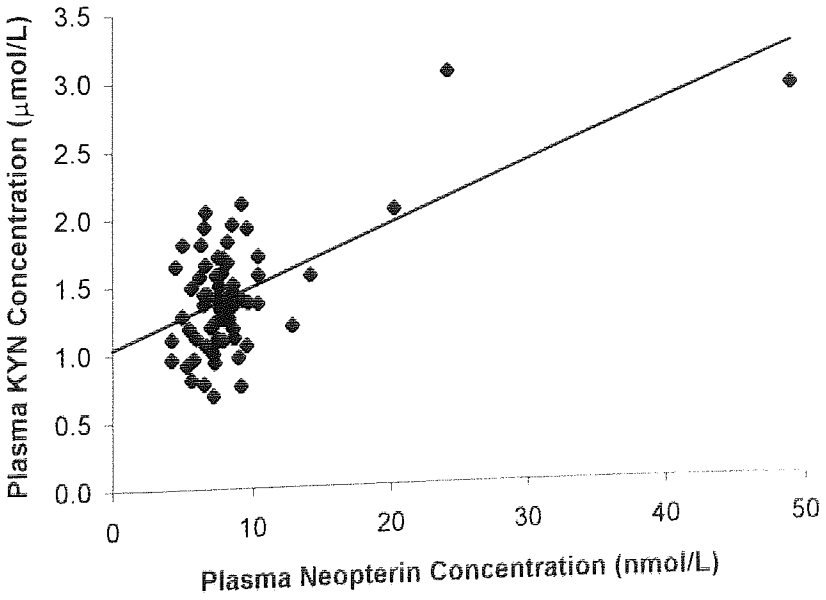

Figure 2.7b. Correlation between plasma KYN and neopterin concentrations in the TS subject group.

(r=0.613; p<0.001, Pearson product-moment correlation coefficient; n=71).

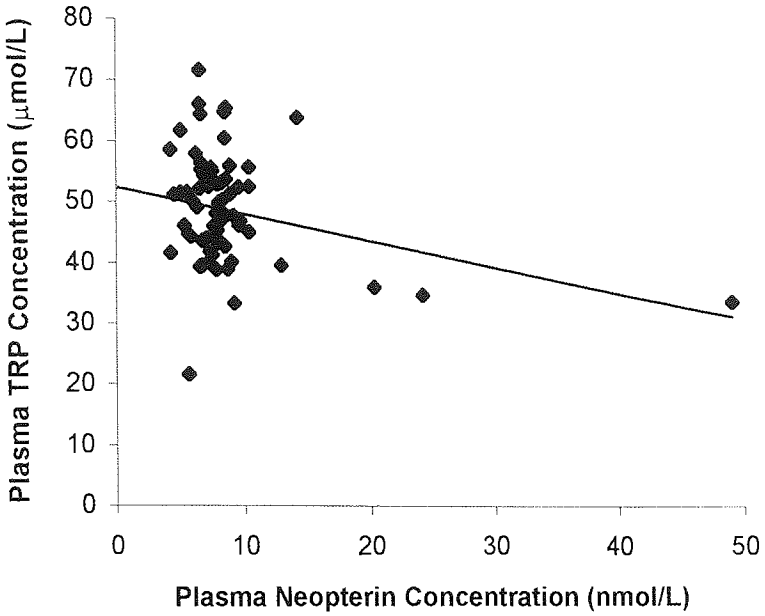

Figure 2.8. Correlation between plasma TRP and neopterin concentrations in the TS subject group.

($r=-0.265$; $p<0.01$, Pearson product-moment correlation coefficient; $n=71$).

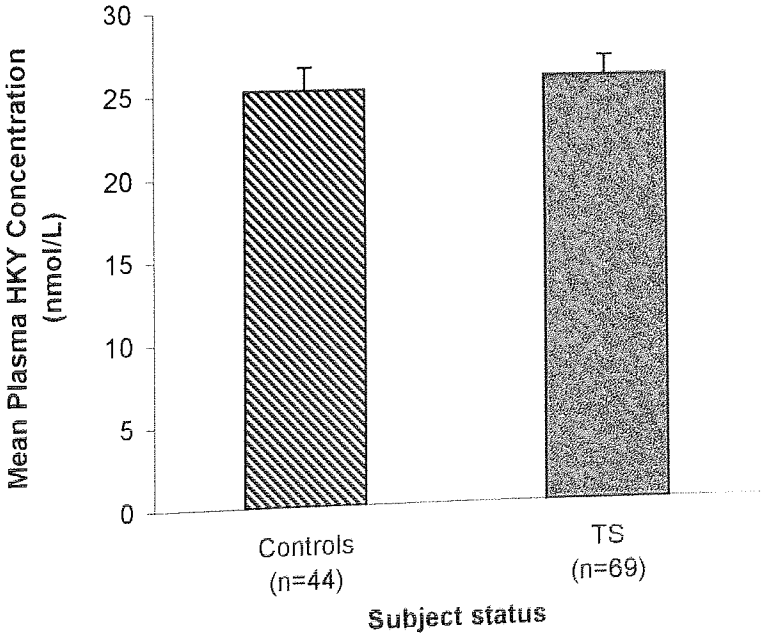

Figure 2.9. Plasma HKY concentrations in TS subject and control groups.

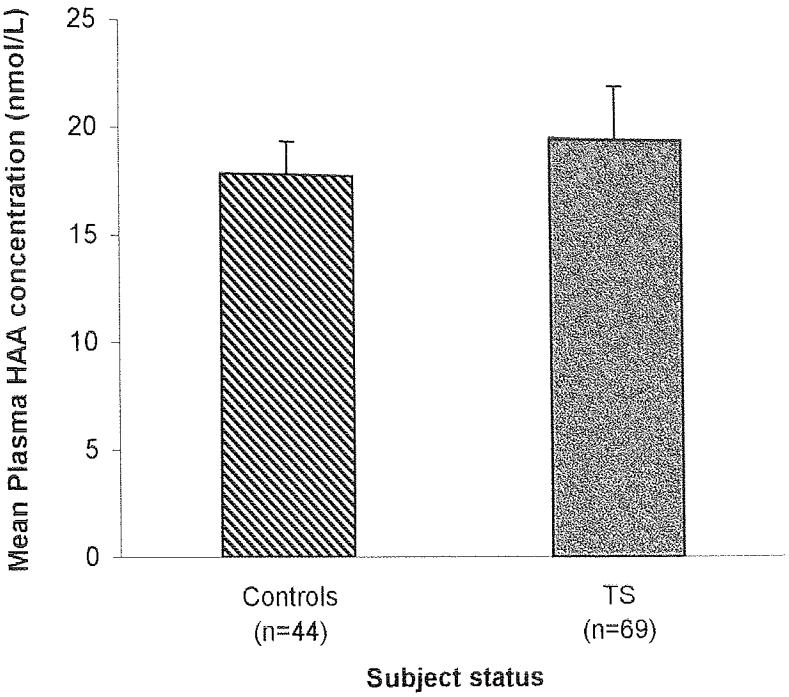

Figure 2.10. Plasma HAA concentrations in TS subject and control groups.

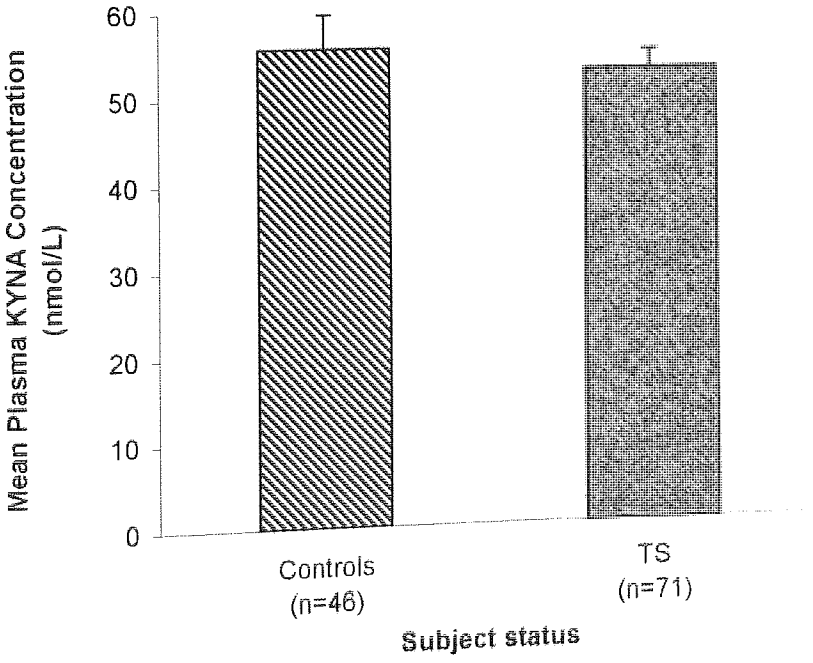

Figure 2.11. Plasma KYNA concentrations in TS subject and control groups.

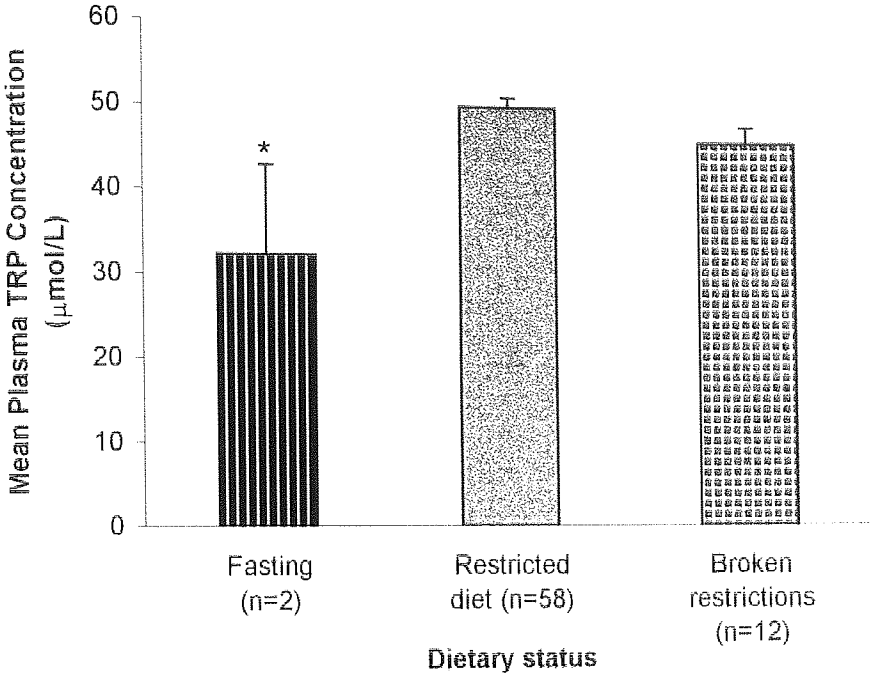

Figure 2.12. Plasma TRP concentration in TS subjects based on dietary status.

*, $p<0.05$ significant difference cf. restricted diet group (Tukey's test).

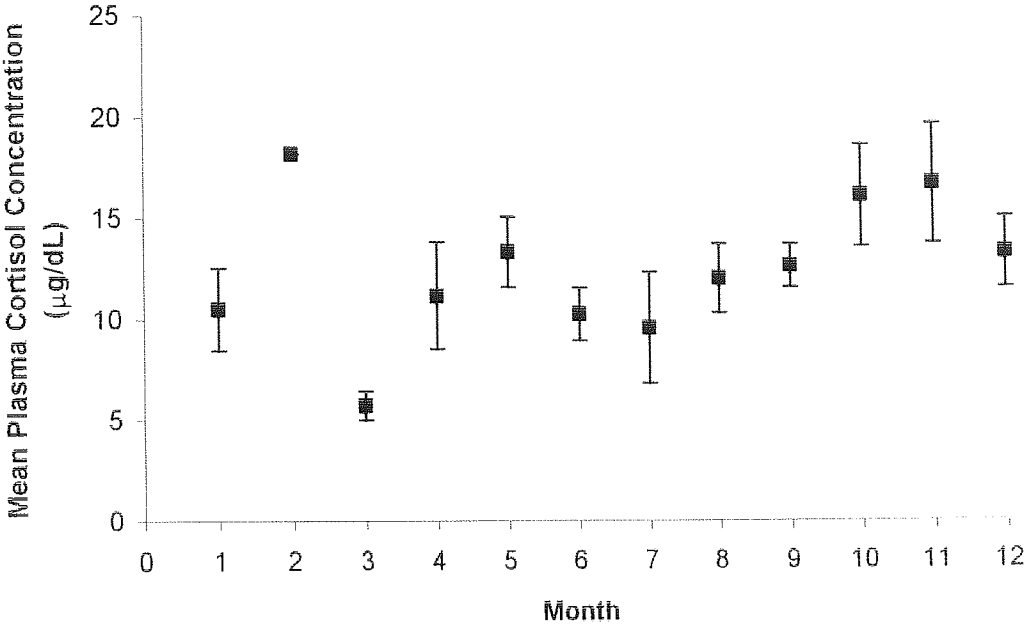

Figure 2.13. Graph to show the change in plasma cortisol concentration during each month for the whole sample group (*i.e.* both TS patients and controls).

Key:

1=July (Controls, n=1; TS, n=3)
2=August (Controls, n=0; TS, n=1)
3=September (Controls, n=0; TS, n=2)
4=October (Controls, n=0; TS, n=9)
5=November (Controls, n=0; TS, n=2)
6=December (Controls, n=0; TS, n=17)
7=January (Controls, n=0; TS, n=4)
8=February (Controls, n=1; TS, n=5)
9=March (Controls, n=30; TS, n=9)
10=April (Controls, n=5; TS, n=7)
11=May (Controls, n=7; TS, n=5)
12=June (Controls, n=2; TS, n=6).

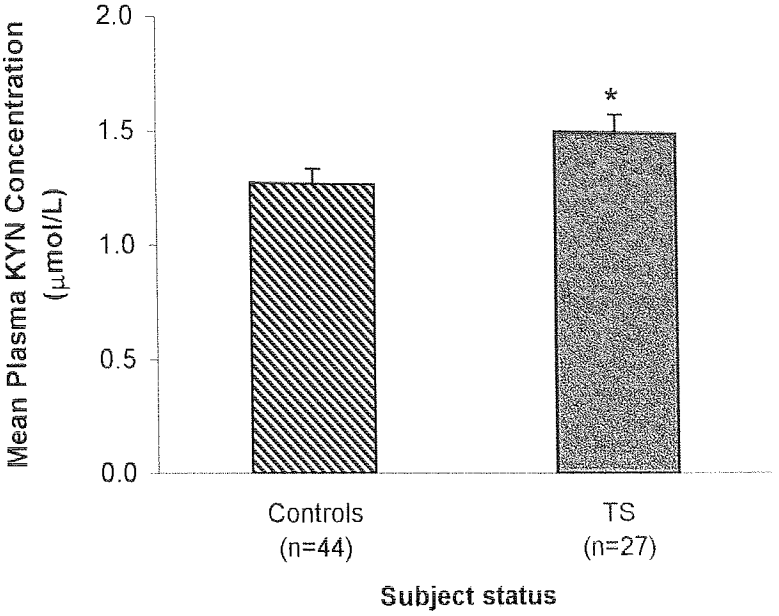

Figure 2.14. Plasma KYN concentrations in TS subject and control groups for samples collected exclusively during months 9 to 12 of the study.

*, $p<0.02$ significant difference cf. controls (Student's t-test; one-tailed).

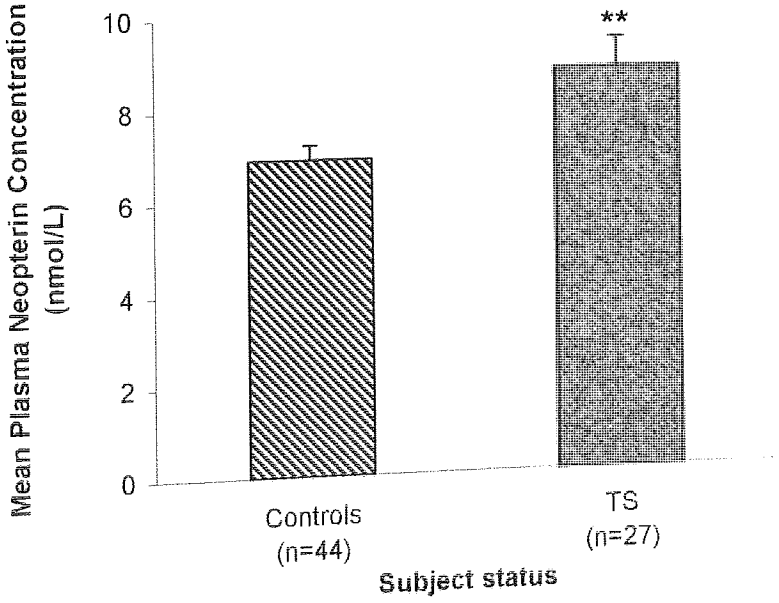

Figure 2.15. Plasma neopterin concentrations in TS subject and control groups for samples collected exclusively during months 9 to 12 of the study.

**, $p<0.01$ significant difference cf. controls (Student's t-test).

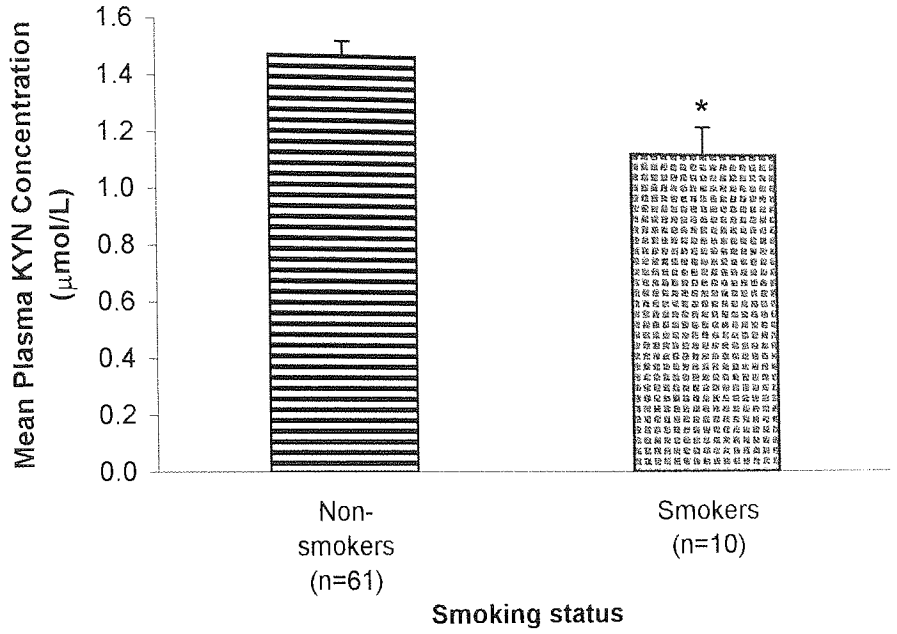

Figure 2.16. Plasma KYN concentrations in TS patients based on smoking status.

*, $p<0.05$ significant difference cf. non-smokers (Student's t-test).

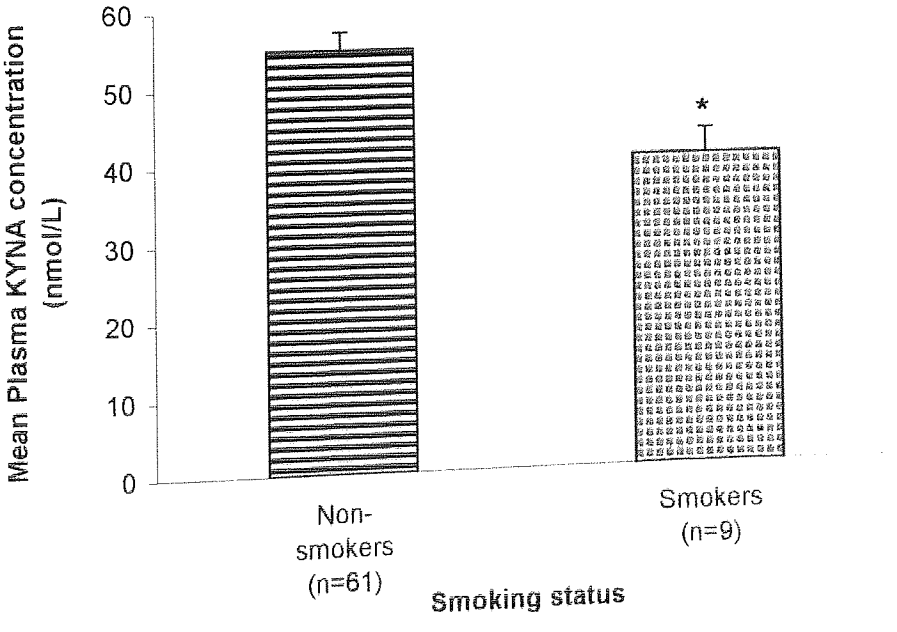

Figure 2.17. Plasma KYNA concentrations in TS patients based on smoking status.

*, $p<0.05$ significant difference cf. non-smokers (Student's t-test).

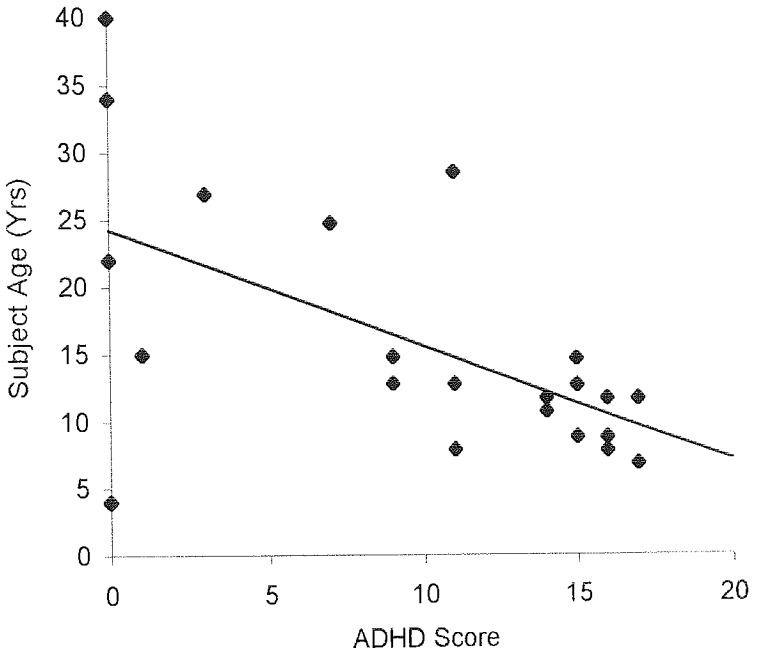

Figure 2.18. Correlation between age and ADHD rating (using DSMIV) in TS subjects.

($r=-0.753$; $p<0.001$, Spearman correlation coefficient; n=23).

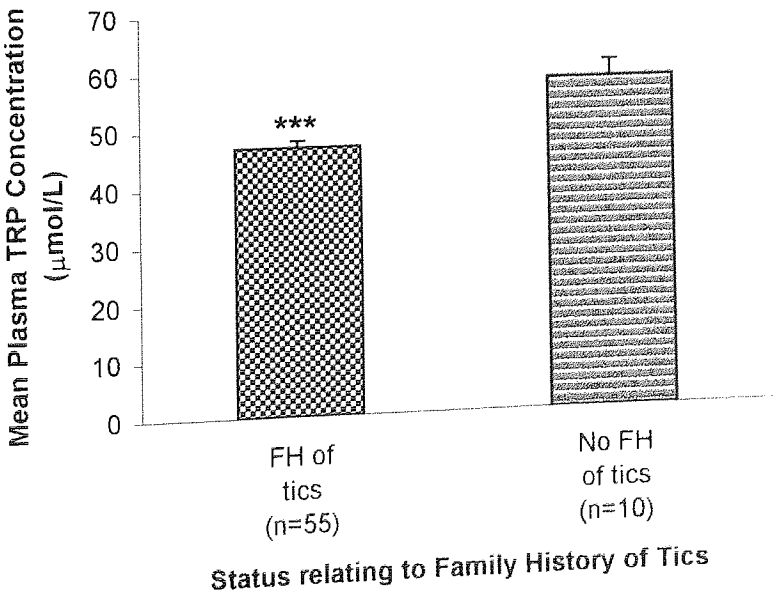

Figure 2.19. Plasma TRP concentrations in TS patients based on whether or not there is a history of tics in the family.

***, $p<0.0001$ significant difference cf. TS patients with no family history of tics (Student's t-test).

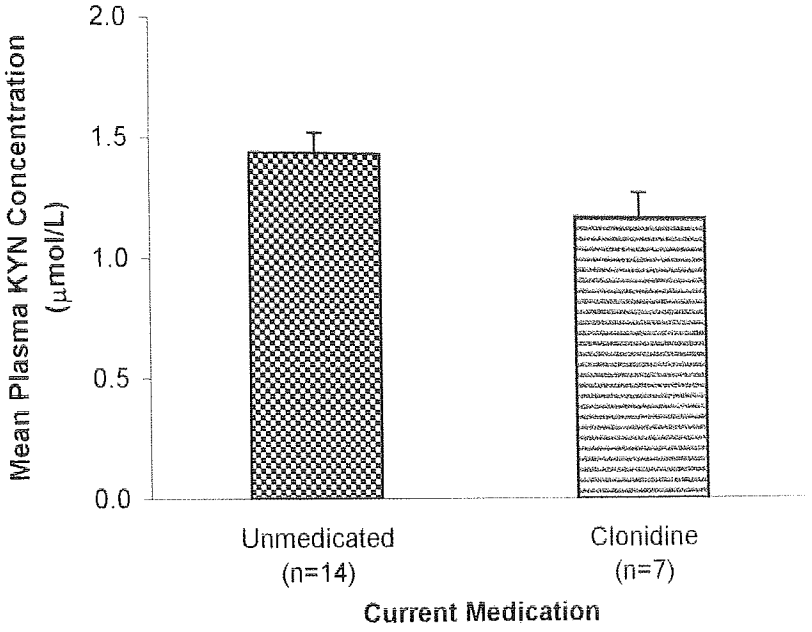

Figure 2.20. Plasma KYN concentrations in TS patients who were either taking clonidine or not taking any medication at the time of the cross-sectional study.

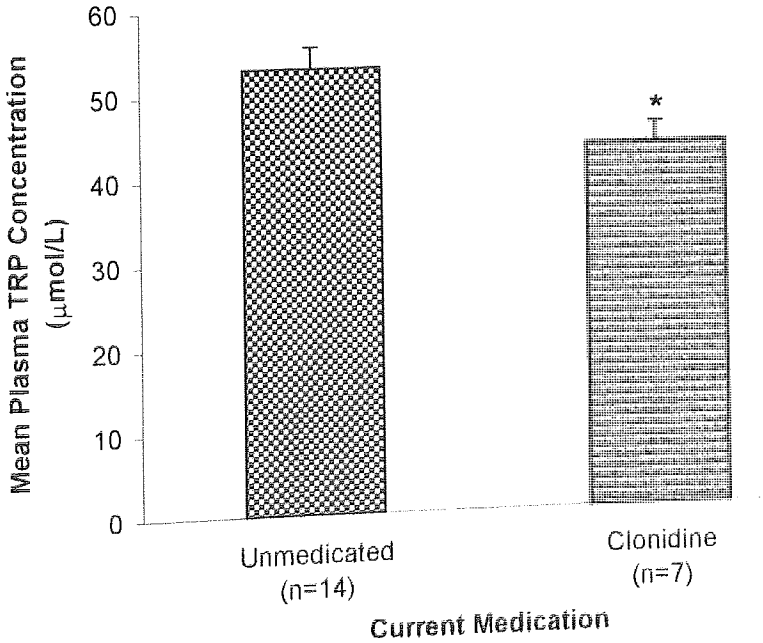

Figure 2.21. Plasma TRP concentrations in TS patients who were either taking clonidine or not taking medication at the time of the cross-sectional study.

*, $p<0.05$ significant difference cf. unmedicated TS patients (Student's t-test).

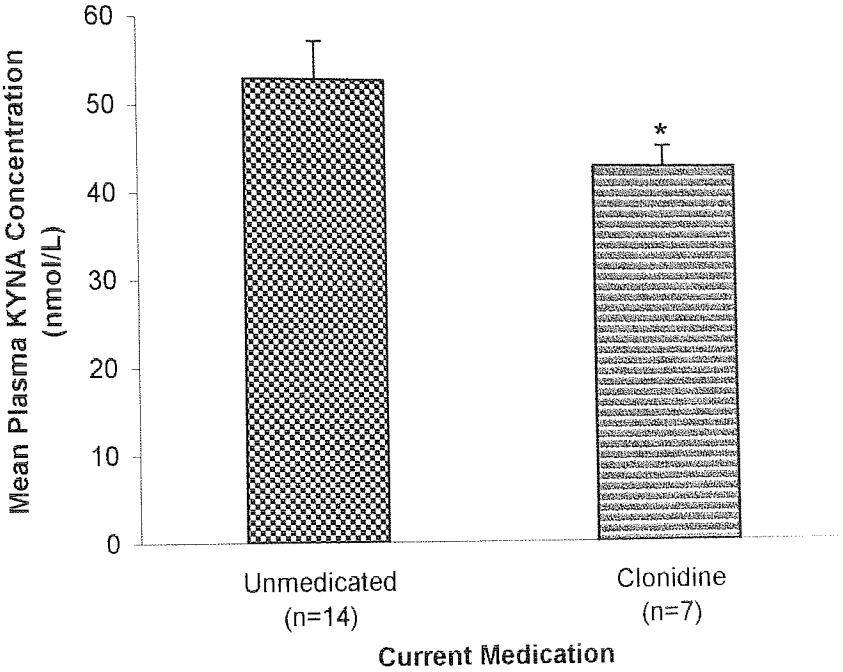

Figure 2.22. Plasma KYNA concentrations in TS patients who were either taking clonidine or not taking medication at the time of the cross-sectional study.

*, $p<0.05$ significant difference cf. unmedicated TS patients (Student's t-test).

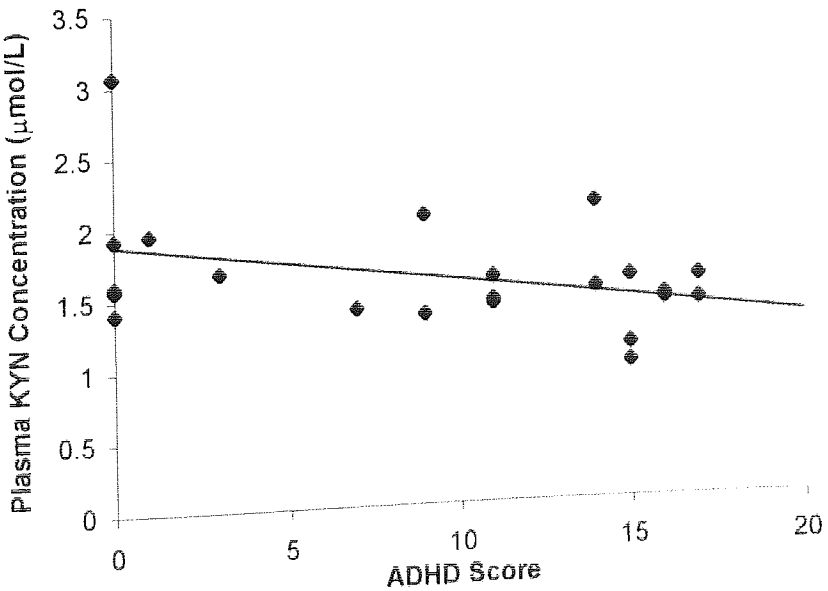

Figure 2.23. Correlation between plasma KYN concentration and ADHD rating (using DSMIV) in TS subjects.

($r=-0.439$; $p<0.05$, Spearman correlation coefficient; n=23).

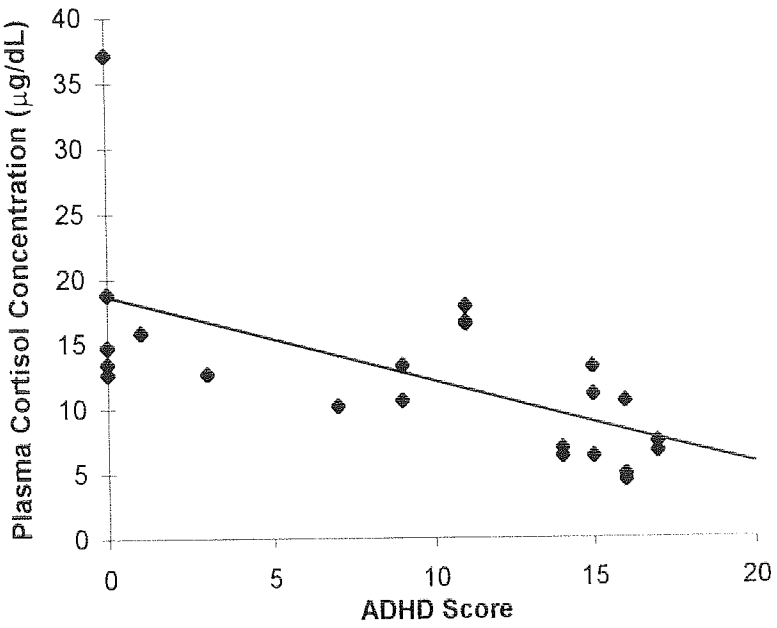

Figure 2.24. Correlation between plasma cortisol concentration and ADHD rating (using DSMIV) in TS subjects.

($r=-0.647$; $p<0.001$, Spearman correlation coefficient; $n=23$).

CHAPTER 3

A LONGITUDINAL STUDY OF TOURETTE'S SYNDROME SYMPTOM SEVERITY IN RELATION TO KYNURENINE PATHWAY VARIABLES AND ANTISTREPTOCOCCAL TITRES.

CHAPTER 3

Introduction

The waxing and waning nature of TS symptoms in patients is a widely recognised phenomenon, with the severity of tics commonly fluctuating in poorly defined cycles, which may last weeks or months. Tics are typically exacerbated by anxiety, anger, emotional stress, excitement and fatigue, and are generally less obvious during sleep, periods of relaxation, concentrating on an enjoyable task or after consumption of alcohol (Robertson, 1989).

Strong evidence has been provided for elevated plasma KYN (Dursun *et al.*, 1994a; also refer to: Chapter 2) and neopterin (see: Chapter 2) in TS patients. Furthermore, given that systemic KYN injection increases 5-HT_{2A}-induced HS in mice (McCreary and Handley, 1995), we have sought to explore the possible relationship between severity of tic symptoms and kynurenine pathway parameters in a group of TS patients, who were monitored over a five month period. An association between TS symptom exacerbation and increased streptococcal antibody titres has also been reported (Swedo *et al.*, 1994), which prompted us to perform ASO titre assays on all samples (subject to availability of plasma).

Additional Methods

Subjects and experimental design

Seven TS patients were recruited to attend the clinic at Queen Elizabeth's Psychiatric Hospital (Birmingham) at 1 month intervals over the same 5 month period. At each attendance, a physical examination was performed to check general health, and the YGTSS was assessed for that particular day by Dr H. Rickards (full details in General Methods). At each visit a blood sample was obtained to enable measurement of plasma TRP, KYN, KYNA, neopterin and cortisol concentrations, and to enable ASO titre assays to be carried out (see: General Methods).

Similar dietary criteria were employed for this investigation to those used in the large cross-sectional study of chapter 2, *i.e.* patients were requested to avoid intake of

aspirin, nicotine and caffeine containing substances from 0:00 hrs of the night preceding collection of blood samples.

Statistical analysis

Clinical rating and biochemical data was compared using the Spearman Correlation coefficient. Relationships between biochemical variables were analysed via the Pearson product-moment correlation coefficient.

Results

Case A

A male subject, age 32 years old, who was taking haloperidol (3mg daily) at the time of month 2 and venlafaxine (75mg daily) on month 4.

No correlation was found between YGTSS and/or any of the biochemical variables (refer to: Table 3.1). Despite the positive readings for the ASO titre assays at months 1 and 3, there did not appear to be any clear effect on the YGTSS on these two occasions.

Case B

A male subject, age 19 years old. The recorded medication for this patient was clomipramine over the first four months; 150mg daily on months 1 and 2, 200mg daily on months 3 and 4.

There was no correlation between YGTSS and the biochemical variables (refer to: Table 3.2), although an association between neopterin and KYNA concentrations was observed (Pearson correlation coefficient, $r=0.878$, $p<0.05$; $n=5$).

Case C

A male subject, age 35 years old. This patient was taking fluoxetine throughout the course of the study, starting at 20mg daily on month 1, which was increased to 40mg daily on month 2, and further elevated to 60mg at month 5.

No correlation was found between YGTSS and/or the biochemical data (refer to: Table 3.3).

Case D

A male subject, age 43 years old, taking no medication.

There was no correlation between YGTSS and/or the biochemical variables (refer to: Table 3.4).

Case E

A male subject, age 21 years old, taking no medication. This patient reported the remains of cold symptoms on month 3.

There was no correlation between YGTSS and/or biochemical data (refer to: Table 3.5), although only four out of six appointments were kept by this subject. However, the somewhat high plasma neopterin concentration found in the month 2 sample is interesting, i.e. greater than three times the size of the next highest value. In the subsequent appointment, the physician was informed that subject E was recovering from cold symptoms, which in light of its role as a marker of immune stimulation, suggests that this could be the source of the elevated neopterin (as well as the slight increase in KYN and cortisol concentrations). It is also noteworthy that positive ASO titres were found for months 1 and 2, and undoubtedly the highest YGTSS for this patient during month 2 of the study.

These results thus invite speculation that the cold symptoms and elevated neopterin are related to infection via streptococci.

Case F

A male subject, age 15 years old, who was taking no medication. This patient reported the symptoms of a cold which was wearing off on month 1.

No correlation was found between YGTSS and the biochemical values (refer to: Table 3.6), but there was an association between the plasma concentrations of TRP and KYN (Pearson correlation coefficient, $r=0.901$, $p<0.05$; $n=6$). Although subject F was recovering from cold symptoms in month 1 of the study, except for a slight increase in plasma neopterin, there was no evidence of any change to the other biochemical variables, ASO titres or YGTSS.

Unfortunately, no YGTSS was submitted on months 5 and 6 for this patient.

Case G

A female subject, age 23 years old, who was taking no medication.

No relationships were found between YGTSS and the biochemical parameters (refer to: Table 3.7). Although plasma KYN and neopterin values appeared to be correlated (Pearson correlation coefficient, $r=0.9998$, $p<0.05$; $n=3$), the significance of this result is questionable, since only three appointments were kept by this patient.

The analysis of all cases together failed to show any association between YGTSS and the kynurenine pathway variables. However, there was a highly significant correlation between plasma KYN and KYNA concentrations (Pearson correlation coefficient, $r=0.560$, $p<0.001$; $n=35$), and also between plasma neopterin and cortisol (Pearson correlation coefficient, $r=0.385$, $p<0.05$; $n=35$).

Discussion

The obvious lack of statistically meaningful data presented in this particular investigation, probably emanated from deficiencies in the design of the study, and due to poor subject reliability in keeping their appointments. The data plainly illustrates how difficult it is to detect possible trends in the biochemical parameters in relation to a symptom rating scale over this short period of time, and with so few data points per subject available for comparison. Certainly, six monthly sets of data per TS patient is the absolute minimum, which would enable anything more than purely speculative observations to be made. Accordingly, since our objective of collecting all the necessary data on each of the six visits was attained with just two members of the study (primarily due to poor patient reliability), meant that any hypotheses would be supported by reflecting on isolated changes to the variables, rather than being based on statistical evidence. In contrast to the problems of poor attendance by subjects in this study, the failure of volunteers to keep appointments in our large cross-sectional investigation (*i.e.* Chapter 2) could be overcome, either by rescheduling the meeting

or recruiting a suitable replacement, since these volunteers were required to donate only one blood sample.

Probably the most interesting result of this longitudinal investigation related to the considerable elevation in plasma neopterin (as well as cortisol) and YGTSS in patient 'case E', which was accompanied by a positive ASO titre reading. This invites speculation of a relationship between immune stimulation via a streptococcal infection and an exacerbation of TS symptoms, although since this was an isolated observation, statistical support is sadly lacking. Nevertheless, there is an obvious opportunity for further exploration of such a hypothesis. One possible direction for further investigation could involve exploitation of the rodent HS model. In a preliminary study carried out within this research by Dr. S. H. Kariyawasam (unpublished data), mice were injected on alternate days with dead streptococci over a period of 3 weeks. Six weeks after the last injection (to allow for stimulation of the immune system) spontaneous and DOI HS frequency were determined and compared to matched controls, but no significant change in HS frequency was found. Nevertheless, modification and expansion of the protocol could prove worthwhile, for example via measurement of biochemical variables (such as KYN and neopterin) and considering alternatives to injecting whole dead streptococci.

As we have already outlined, any future human studies of a correlation between TS symptom severity and specific biochemical variables must be considered thoroughly. Ideally, the timespan of these investigations and the number of visits made by each TS patient over this period should be increased, although unfortunately the reliability of patients to keep all appointments cannot be guaranteed.

Tables 3.1. to 3.7. Clinical ratings and biochemical data for each TS patient corresponding to their monthly clinic appointments for the sequential study.

(n/a=non-attendance by patient; n/s=no score obtained by physician; i/p=insufficient plasma available).

Table 3.1. *Case A*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	38	1.04	49.3	56.1	7.7	21.31	+ve
2	37	1.61	42.8	52.6	7.5	16.46	-ve
3	25	1.63	42.7	55.1	8.6	21.33	+ve
4	25	1.08	33.5	38.0	9.1	14.52	-ve
5	22	1.75	43.5	62.6	7.8	23.55	-ve
6	32	1.42	45.5	40.1	8.9	21.94	-ve

Table 3.2. *Case B*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	26	1.10	53.4	27.7	6.2	19.93	-ve
2	30	1.03	51.2	23.3	5.2	15.88	-ve
3	32	1.12	65.2	34.6	8.7	16.19	-ve
4	26	1.23	58.0	35.2	6.6	11.30	-ve
5	30	1.14	52.2	40.0	9.1	13.17	-ve
6	n/a						

Table 3.3. *Case C*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	34	1.26	45.6	54.0	6.1	8.56	-ve
2	31	0.89	57.0	47.5	6.3	18.33	-ve
3	36	1.24	51.8	35.2	4.7	11.44	-ve
4	44	1.36	50.5	68.3	6.7	19.08	-ve
5	37	1.38	62.7	53.5	8.6	18.93	-ve
6	45	1.28	57.2	53.7	6.3	14.11	-ve

Table 3.4. *Case D*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	30	1.32	50.3	53.8	6.8	13.20	-ve
2	36	1.36	54.6	41.4	5.3	13.26	-ve
3	31	1.35	49.5	46.5	4.7	16.09	-ve
4	34	1.62	49.6	47.4	6.6	15.33	-ve
5	n/a						
6	32	1.36	52.2	51.8	7.3	11.14	-ve

Table 3.5. *Case E*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	18	1.26	54.6	51.8	7.5	19.61	+ve
2	29	1.66	53.8	47.8	27.4	25.14	+ve
3	12	1.61	51.7	48.5	6.7	10.55	-ve
4	11	1.37	55.5	53.2	8.1	14.86	-ve
5	n/a						
6	n/a						

Table 3.6. *Case F*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	40	1.50	49.9	69.0	8.7	12.45	-ve
2	35	1.27	45.1	50.3	6.4	9.40	-ve
3	51	0.75	39.6	26.4	7.0	13.41	-ve
4	51	0.86	37.7	34.5	8.0	14.30	i/p
5	n/s	1.61	60.6	46.4	5.4	26.22	i/p
6	n/s	0.89	43.4	44.6	7.7	13.86	i/p

Table 3.7. *Case G*

Month	YGTSS	KYN (μmol/L)	TRP (μmol/L)	KYNA (nmol/L)	Neopterin (nmol/L)	Cortisol (μg/dL)	ASO
1	26	1.01	50.0	44.4	7.0	12.22	i/p
2	n/a						
3	10	1.16	46.8	40.8	8.4	16.18	i/p
4	n/a						
5	16	1.33	36.3	43.4	10.1	24.00	i/p
6	n/a						

CHAPTER 4

THE EFFECTS OF NICOTINE ON TRYPTOPHAN PATHWAY METABOLITES AND ON HEAD-SHAKES IN MICE.

CHAPTER 4

Introduction

For many years it has been known that head-shakes (HS) and wet-dog shakes (WDS) occur in rodents both spontaneously and following administration of agents which increase 5-HT activity. It is widely accepted that these movements are mediated predominantly by 5-HT_{2A} receptors, initially based on the evidence of studies showing that ritanserin (a specific 5-HT_{2A} receptor antagonist) reduced tics induced by morphine and clonidine withdrawal in mice (Handley et al., 1986). Further support was rendered by Kennett and Curzon (1991) who found a significant correlation between the ID50 values of ten antagonists against 5-HTP + carbidopa-induced HS and their affinities for 5-HT_{2A} receptors, but no such correlation was found for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} (now referred to as 5-HT_{2C}) or 5-HT_{1D} receptors. Experiments have also shown that drugs with activity at the 5-HT_{1A} receptor site, modulate rodent head twiches induced by the selective 5-$HT_{2A/2C}$ agonist DOI (Dursun and Handley, 1993; Schreiber *et al.*, 1995).

In mice, systemic administration of both KYN and its metabolite HKY potentiated the HS response provoked by centrally administered 5-HT or systemic 5-HTP (Handley and Miskin, 1977). Moreover, McCreary and Handley (1995) reported that KYN potentiates the frequency of HS induced by DOI, although KYN alone had no effect on spontaneous HS. However, while the 5-HT_{2A} receptor is accepted as being the likely source of this shaking behaviour, ligand binding studies have shown that neither KYN nor its metabolites bind to 5-HT_{2A} or 5-HT_{1A} receptors (Kariyawasam *et al.*, 1997). Thus, the precise mechanism by which KYN (and/or its metabolites) influence these shaking behaviours remains uncertain.

In agreement with the report by Dursun *et al.* (1994a), we detected a significant increase in the plasma KYN concentrations of TS patients compared with matched controls (refer to: Chapter 2). In addition, we found a difference in plasma KYN between smoking and non-smoking TS subjects, with smokers exhibiting significantly lower KYN levels compared with the non-smokers. Analysis of what effect smoking status incurred on other kynurenine pathway substances revealed a similar pattern in

plasma KYNA, although TRP, HKY and HAA were unaffected. In view of these findings, we decided to investigate how various nicotine treatment regimens might influence the levels of some kynurenine pathway metabolites in mouse plasma, as well as brain KYN concentrations.

Recent clinical studies have demonstrated that nicotine may be beneficial in ameliorating tic symptoms either on its own (Silver and Sanberg, 1993; Dursun *et al.*, 1994b) or in combination with haloperidol (Sanberg *et al.*, 1989; McConville *et al.*, 1991). The rodent head twitch model is a useful pharmacological instrument, which could provide an insight into how nicotine might influence these tic symptoms. Since it is well established that the 5-HT_{2A} receptor plays a central role in mediating the HS response, it is possible that any nicotine effects on shaking behaviour would be manifested via changes in 5-HT turnover in the rodent brain. This provided an additional avenue for exploring the possible interactions between these biochemical pathways.

The purpose of the current study was to investigate the effects of nicotine on spontaneous and DOI-induced HS in mice, following acute and chronic nicotine administration. Measurement of kynurenine pathway substances in mouse plasma, and biochemical assessments of whole brain 5-HT turnover, as well as brain KYN concentrations, were carried out to examine the effects of these nicotine treatments on tryptophan metabolic pathways. The nicotinic receptor antagonist mecamylamine was used to help verify the mechanism by which acute nicotine may modify DOI-HS. In addition, the effects of the novel potent nicotinic agonist (±)-epibatidine (Badio and Daly, 1994; Sullivan *et al.*, 1994) on DOI-HS (acute treatment only) was investigated. (±)-Epibatidine was selected because it exhibits binding to peripheral and central nicotinic receptor sites and like nicotine it can also penetrate the brain (Khan *et al.*, 1997; Prof. Ian P. Stolerman, personal communication). Therefore, use of (±)-epibatidine might further clarify whether any nicotine-induced changes in shaking behaviour were mediated via a direct action at nicotinic cholinergic receptors.

Additional Methods

Subjects and Experimental design

(a) Acute studies:

In experiments where the effect of acute (–)-nicotine (0.4, 0.8 or 1.6mg/kg s.c.) or (±)-epibatidine (2.5, 5.0, 10μg/kg s.c.) on spontaneous or DOI-induced HS was examined, the nicotinic receptor agonist (or saline vehicle) was injected 10 minutes before measurement of HS frequency. When monitoring the blocking action of mecamylamine (1mg/kg s.c.) on these effects, mice were pretreated with mecamylamine a further 20 minutes prior to injection with nicotine, epibatidine or saline vehicle.

(b) Drug treatment paradigms for chronic (7 days) nicotine studies:

Mice were randomly assigned to one of four treatment groups. Two groups of animals received a s.c. injection of either (–)-nicotine (1.6 mg/kg) or saline twice daily (09:00 and 18:00 hrs) for 7 days. The remaining two groups of mice were treated with a single dose of either nicotine (1.6 mg/kg) or saline, which was administered at 18:00 hrs on the final day of the dosing regimen.

The 7 days control group was necessary to negate the effects produced by the injections or by repeated handling of the animals. Housing the acutely treated groups in cages in the same room as those mice treated chronically for the entirety of the experiment helped account for variations in environmental factors, such as noise, temperature and smells.

The experimental protocol described for chronic nicotine (7 days) effects on spontaneous and DOI-induced HS was identical. The one exception was that mouse brain and plasma assays were carried out only in the experiments relating to spontaneous HS (see below), because changes in biochemical variables after measurement of DOI-induced HS could not be assumed to emanate purely from the nicotine treatment.

Subsequent handling of chronically treated mice

(a) Spontaneous HS response:

On the morning of day 8, mice were divided into observation aquaria containing one animal from each treatment group, and spontaneous HS frequency was assessed (refer to: General Methods). Immediately afterwards, blood was collected by means of cardiac puncture under general anaesthesia, and the animals were then decapitated to enable removal of the brains, which were dissected to remove the cerebellum. The dissected brains and plasma obtained via centrifugation of whole blood samples (see: General Methods), were stored at −70°C until being assayed. Whole brain concentrations of 5-HT and 5-HIAA, as well as plasma and brain KYN concentrations were determined by HPLC (as described in: General Methods). The ratio of 5-HT/5-HIAA provided a crude measure of 5-HT turnover in the whole brains of the mice.

Collection of blood by cardiac puncture was kindly carried out by Mr M. Gamble.

(b) DOI-induced HS response:

On the morning of day 8, mice being assessed for the effects of single dose and chronic nicotine treatment on DOI-induced HS were divided into observation aquaria in a random fashion, each containing 4 animals. The individual groups of mice were then injected with DOI (0.5mg/kg i.p.), and DOI-induced HS frequency was quantified (refer to: General methods).

Measurement of brain and plasma KYN after acute nicotine treatment

To investigate how brain and plasma KYN levels may correlate with changes in HS frequency after acute nicotine (1.6mg/kg; 10 mins pretreatment time) treatment, brain and plasma were obtained as before, and KYN concentrations were measured for each sample (see: General Methods).

Measurement of kynurenine pathway metabolites in mouse plasma following chronic nicotine treatment

To investigate the effects of chronic nicotine treatment on kynurenine pathway substances, two groups of 6 mice were injected twice daily with nicotine (1.6mg/kg s.c.) or saline for 7 days, as detailed previously. Blood was collected on day 8 by cardiac puncture, and the resulting plasma stored at –70°C until being assayed. HPLC was used to measure the plasma concentrations of TRP, KYN, KYNA, HKY and HAA (refer to: General Methods).

Behavioural tests and collection of blood and brain samples were carried out between 9:00 and 12:00 hrs to minimise the possible effects of diurnal variation.

Statistics

All values presented are means ± SEM. A Student's *t*-test for unpaired data was used to determine differences between two groups of treatment. Differences between multiple groups were compared using a one-way ANOVA as a function of drug treatment, and following significant effects, a *post-hoc* Duncan's test was performed. Data was also compared where appropriate using correlational analysis.

Results

Effects of acute nicotine (10 mins pretreatment time) on DOI-HS

DOI-induced HS were dose-dependently reduced by acute nicotine treatment (dose range 0.4 to 1.6mg/kg), which proved significant at the 1.6mg/kg dose [$F_{(3, 36)}=3.38$, $p<0.03$; *post hoc* test $p<0.05$] as shown in Figure 4.1.

The centrally acting nicotinic receptor antagonist mecamylamine (1mg/kg) completely blocked the effect of nicotine (1.6mg/kg) [$F_{(3, 28)}=3.24$, $p<0.05$; *post hoc* test $p<0.05$] as displayed by Figure 4.2. Mecamylamine alone had no effect on the DOI-HS response.

In addition, it was on the basis of these results that the 1.6mg/kg nicotine dose was selected for comparison of single dose and chronic nicotine treatments on shaking behaviours and biochemical variables.

Effects of acute epibatidine (10 mins pretreatment time) on DOI-HS

DOI-induced HS were dose-dependently reduced by acute epibatidine treatment (dose range 2.5 to 10.0μg/kg), which was significant at the doses 5.0 and 10.0μg/kg compared with the lowest dose of epibatidine and saline [$F_{(3, 20)}$=15.94, $p<0.0001$; *post hoc* test $p<0.05$] as shown in Figure 4.3.

Mecamylamine (1mg/kg) abolished the effect of epibatidine (10.0μg/kg) on DOI-HS [$F_{(3, 20)}$=30.60, $p<0.0001$; *post hoc* test $p<0.05$]. Mecamylamine alone had no effect on the DOI-HS response (see: Figure 4.4).

Effects of acute nicotine on spontaneous HS

Acute administration of nicotine (1.6mg/kg, 10 mins pretreatment time) to mice had no effect spontaneous HS frequency (see: Figure 4.5).

Effects of acute nicotine on plasma and brain KYN

Acute nicotine treatment (1.6mg/kg, 10 mins pretreatment time) had no effect on plasma or brain KYN concentrations (refer to: Table 4.1). However, there was a highly significant correlation between plasma and brain KYN for both saline (n=6; r=0.983, $p<0.001$, Pearson product-moment coefficient) and nicotine (n=6; r=0.950, $p<0.001$, Pearson product-moment coefficient) treated mice (see: Figures 4.6a and 4.6b respectively). It is noteworthy, that although the KYN data from the saline treated mice demonstrates a highly significant linear correlation, close inspection of the plotted data shows that the points appear to trace the shape of a curve (see: Figure 4.6a).

Effects of chronic nicotine on DOI-HS

Mice treated twice daily with nicotine (1.6mg/kg) for 7 days showed a significant increase in DOI-induced HS frequency, compared with either chronic saline treatment or the single nicotine (1.6mg/kg) or saline injections [$F_{(3, 20)}$=6.48, $p<0.01$; *post hoc test* $p<0.05$] (refer to: Figure 4.7).

Effects of chronic nicotine on spontaneous HS

The chronic nicotine group showed a significant increase in spontaneous HS compared to either single dose or chronic saline treatments [$F_{(3, 32)}=3.81$, $p<0.03$; *post hoc test* $p<0.05$] (see: Figure 4.8).

Effects of chronic nicotine on plasma and brain KYN and 5-HT turnover in mouse brain

Measurement of 5-HT and 5-HIAA whole brain levels and calculation of 5-HT turnover (*i.e.* 5-HT/5-HIAA ratio) showed no difference as a consequence of chronic nicotine (1.6mg/kg) treatment (refer to: Table 4.2). However, there was a significant elevation of plasma KYN [$F_{(3, 20)}=3.59$, $p<0.05$; *post hoc* test $p<0.05$] and brain KYN [$F_{(3, 20)}=5.27$, $p<0.01$; *post hoc* test $p<0.05$] following the 7 day nicotine schedule (Table 4.2). No correlation was found between spontaneous HS frequency and either plasma or brain KYN (using Spearman correlation coefficient). Furthermore, central and peripheral KYN was significantly correlated, but only when data from all treatment groups was analysed together ($n=24$; $r=0.71$, $p<0.001$, Pearson product-moment coefficient).

Effects of chronic nicotine on kynurenine pathway metabolites in mouse plasma

The results from this experiment (summarised in Table 4.3) confirmed the earlier reported increase in plasma KYN after 7 days twice daily systemic administration of nicotine (Nic)(1.6mg/kg) compared with saline (Sal) treated mice (Sal=1.26 ± 0.18, Nic=1.80 ± 0.11, μmol/L; $p<0.05$). Although nicotine treated mice showed a trend towards increased plasma TRP (Sal=58.0 ± 4.2, Nic=70.5 ± 3.9, μmol/L; $p=0.052$) and KYNA (Sal=52.1 ± 9.2, Nic=72.7 ± 5.8, nmol/L; $p=0.088$), this effect was not significant. On the other hand, there was no evidence of a nicotine influence on plasma HKY or HAA levels (refer to: Table 4.3). Finally, there was a weak correlation between KYN and TRP concentrations using the combined data from nicotine and saline groups ($n=12$; $r=0.58$, $p<0.05$, Pearson product-moment coefficient).

Discussion

The dose dependent decrease in DOI-induced HS frequency following acute nicotine injection neatly conforms with the clinical reports of tic symptom improvement in TS patients treated with nicotine gum or transdermal patches either alone (Dursun *et al.*, 1994b) or concurrently with neuroleptics (Sanberg *et al.*, 1989; McConville *et al.*, 1991; Silver and Sanberg, 1993; Silver *et al.*, 1996). The comparable effects of epibatidine on DOI-HS in mice, and the blocking action of the central nicotinic receptor antagonist mecamylamine on these rodent shaking behaviours, suggests that this effect is mediated at the nicotinic cholinergic receptor level.

Inhibition of DOI-HS by acute nicotine treatment compares favourably with the findings of Shytle *et al.* (1995), who reported an attenuation of kainic acid-induced WDS in rats pretreated with nicotine. Shytle and colleagues speculated that the mechanism of this effect could be via a GABA-mediated process, since GABA agonists consistently inhibit behavioural and neurotoxic consequences of kainic acid administration (Lenicque *et al.*, 1979; Worms *et al.*, 1981). Further support emanates from reports that nicotine can induce the spontaneous release of GABA from hippocampal synaptosomes (Wonnacott *et al.*, 1989). Interactions between the nicotinic cholinergic and glutamatergic systems have also been studied (Shoaib *et al.*, 1994; Aizenman *et al*, 1991). In one report, nicotine was found to both partially inhibit whole-cell NMDA induced responses and displace the non-competitive NMDA receptor antagonist, MK-801 (Aizenman *et al.*, 1991). In addition, Grenningloh *et al.* (1987) reported on the existence of structural homologies between nicotinic acetylcholine receptors and glycine receptors, which prompted Shytle *et al.* (1995) to contemplate that nicotine may interact directly with the NMDA receptor complex. However, given the major role of serotonergic systems in animal shaking behaviours (see: General Introduction), it is also worthwhile considering the possible significance of serotonergic pathways. Indeed, Worms *et al.* (1981) observed that WDS in rats induced by the glutamate analogue kainic acid were potentiated by compounds which enhance serotonergic transmission, whereas substances possessing 5-HT blocking properties caused a decrease in WDS.

In contrast to the acute effects of nicotine, the data from the chronic (7 days) nicotine treated mice yielded an unexpected series of results. First of all, the marked elevation in both peripheral and central KYN concentrations after chronic nicotine, seems contradictory to those observed in the human clinical study (refer to: Chapter 2), in which TS smokers had significantly lower plasma KYN than TS non-smokers. This apparent conflict between the animal and human KYN data was complicated by the potentiation of spontaneous and DOI-induced HS in chronic nicotine treated mice, which challenges the concept of nicotine's beneficial properties in human tic disorders. Hence, it seems appropriate to consider whether mecamylamine would inhibit the effects of chronic nicotine on shaking behaviour and KYN concentrations. However, there is the question of what stage of the experiment would be best to administer the nicotinic antagonist. For example, mecamylamine could be given shortly before each nicotine injection, prior to measurement of HS frequency or alternatively at both of these stages of the investigation.

A possible explanation for the contrasting behavioural results generated by acute and chronic nicotine treatment could be via changes in the sensitivitiy and/or number of receptors which mediate these responses. A number of studies have shown an up-regulation of nicotinic binding sites in the CNS following nicotine injections and infusion (Marks and Collins, 1985; Marks *et al.*, 1985; Schwartz and Kellar, 1985). Marks and colleagues (1985) reported increased [^{3}H]-nicotine binding in the mouse following slow continuous infusion of nicotine at a dose of 4mg/kg/hr, which proved maximal after 4 days of dosing. The greatest increases in [^{3}H]nicotine binding occurred in the thalamus and the smallest in the midbrain area. This increased binding was accompanied by a parallel development of tolerance to the effects of nicotine on Y-maze activity and rears, body temperature and heart rate. Marks and coworkers therefore speculated that specific brain areas may be primarily responsible for regulating specific nicotine-induced responses, and a closer examination of these brain areas could reveal the extent of the relationship between drug response and brain nicotinic receptors. In agreement with this, Schwartz and Kellar (1985) reported an increase in [^{3}H]ACh binding to nicotinic receptors after 5 days of subcutaneous nicotine treatment, reaching a maximal increase after 10 days. In the

same study, chronic treatment with the cholinesterase inhibitor diisopropyl flurophospate, which would be expected to raise endogenous acetylcholine levels, caused a reduction in [^{3}H]ACh binding in the rat brain. This latter result is consistent with the theory of agonist-induced down-regulation of the nicotinic receptor site. The apparent incongruity of these agonist effects might be related to the powerful desensitising properties of nicotine, so that on prolonged exposure it acts functionally as an antagonist rather than an agonist (Marks *et al.*, 1985, Wonnacott, 1987). In contrast with these studies, nicotine given to rats in their drinking water for 41 weeks resulted in an increase in [^{3}H]nicotine binding sites in the cortex, whereas levels in the hippocampus were decreased (Nordberg *et al.*, 1985). Consequently, the concentration and route of administration is likely to be of crucial importance to the induction of such changes.

Up-regulation of [^{3}H]nicotine binding sites has also been observed in the brains of cigarette smokers. Comparison of post-mortem brain samples from smokers and non-smokers in one study showed significant increases in the number of [^{3}H]nicotine binding sites in Ammon's Horn, lateral hippocampal gyrus, gyrus rectus and cerebellum (Wonnacott, 1987).

The significance of potential alterations in nicotinic cholinergic receptors and changes in shaking behaviour after chronic nicotine treatment is not clear, although it is well documented that activation of nicotinic cholinergic receptors leads to facilitation of the release of a number of neurotransmitters, including acetylcholine, dopamine, noradrenaline, 5-HT, GABA and glutamate (reviewed by: Brioni *et al.*, 1997). In a review by Wonnacott (1987) it was estimated that in certain brain areas, a substantial proportion of nicotine receptor sites (30%) occur presynatically, which correlates well with nicotine's ability to facilitate transmitter release from nerve terminals. For example, it was reported that systemically administered nicotine increases extracellular 5-HT levels (measured by *in vivo* dialysis) in the frontal cortex of anaesthetised and awake rats (Ribeiro *et al.*,1993). Furthermore, increased extracellular levels of noradrenaline (Brazell *et al.*, 1991) and dopamine (Benwell *et al.*, 1992) were detected following acute systemic nicotine treatment. Thus nicotine could theoretically produce behavioural changes both directly via activation of

nicotinic cholinergic receptors and indirectly via transmitter systems other than acetylcholine. The association of nicotinic cholinergic receptors with a vast range of neuronal pathways and neurotransmitters, could lead to subtle presynaptic effects, as well as a postsynaptic action.

The probable heterogeneity of nicotinic receptor sites is an additonal factor to consider. Ligand binding studies suggest that three subclasses of nicotinic cholinergic receptors occur in the brain: (1) the high-affinity binding site for [^{3}H]nicotine, (2) the lower affinity binding site, which has a high affinity for the potent neuromuscular antagonist α-bungarotoxin, and (3) a population of receptors that show selectivity for neuronal bungarotoxin (see reviews by: Stolerman *et al.*, 1995; Brioni *et al.*, 1997). It is thus feasible that variations in the binding characteristics of nicotine to [^{3}H]nicotine and/or α-bungarotoxin binding sites in the brain may lead to the mediation of biochemical and behavioural changes.

The HS response has itself been shown to be modified by a wide range of substances, possessing diverse pharmacological profiles (see: Handley and Singh, 1986a), and so predicting exactly how nicotine might effect the relevant biochemical changes is an enormous task. The DOI-induced HS (and spontaneous HS) response is widely accepted as being mediated via the 5-HT$_{2A}$ receptor (refer to: General Introduction). Therefore, the influence of nicotine on 5-HT turnover seemed an appropriate angle from which to tackle this question. No changes in 5-HT, 5-HIAA or 5-HT turnover were detected in mouse whole brains after chronic nicotine administration in this study. However, Benwell and Balfour (1979) reported that groups of rats injected with nicotine (0.4mg/kg) for various lengths of time (up to a maximum period of 40 days), consistently exhibited reduced hippocampal concentrations of 5-HT, and reduced 5-HIAA to a lesser extent. In contrast, there was no significant difference in 5-HT or 5-HIAA in the hypothalamus or in residual brain samples. Hence, we can only speculate on the possible benefits of carrying similar assays on discrete brain areas for future experiments, as opposed to analysing whole brains. More recently, Benwell and Balfour (1982) reported a decrease in the rate of formation of 5-HT in synaptosomes derived from the hippocampus brain region of rats following acute and chronic (40 days treatment) administration of nicotine (0.4mg/kg s.c.). Moreover,

chronic nicotine treatment reduced the rate of *L*-TRP uptake by hippocampal synaptosomes, which appeared to be the result of a decrease in the number of amino acid carrier molecules in the nerve terminal membrane, rather than an alteration in the affinity of the carrier for TRP. They suggested that the selective decrease in 5-HT activity in the hippocampus of rats treated chronically with nicotine was due to reduced 5-HT biosynthesis in this brain region. However, it is also possible that any TRP deficit will lower the activity of the kynurenine pathway.

Evidence from human postmortem studies are supportive of nicotine's potential to evoke regionally-selective reductions in the levels of 5-HT and its principal metabolite in the brain. Balfour (1989) reported that tissue taken from brains of subjects who had smoked regularly (for more than two years prior to death), showed a significantly lower concentration of 5-HT in the hippocampal formation, compared with the levels found in age-matched non-smoking controls. The concentrations of 5-HIAA in the hippocampal formation and the hippocampal neocortex showed a similar deficit in the brains of smokers, as did the median raphe nuclei, which provides the main serotonergic innervation to the hippocampus.

Data from binding studies indicated that smoking was associated with increased binding of ligands to 5-HT_1 and 5-HT_{1A} receptors in the hippocampal cortex, as well as an up-regulation of 5-HT_{1A} receptors in the hippocampal formation (Balfour, 1989). However, no change in [^{3}H]ketanserin binding in any of the brain regions was observed. This data is thus consistent with a nicotine related reduction in 5-HT turnover in the hippocampus.

Some research groups have demonstrated that nicotine administration in rats induces a dose-dependent increase in plasma corticosterone levels, which is mediated by nicotinic receptors originating from within the CNS (Benwell and Balfour, 1979; Pauly *et al.*, 1992). It has also been found that rats rapidly develop tolerance to this stimulatory effect on corticosterone secretion when treated chronically with nicotine (Benwell and Balfour, 1979). However, if the plasma corticosterone levels are determined 24 hours after the last nicotine injection, elevated corticosterone is consistently exhibited in nicotine treated animals compared with matched controls (Benwell and Balfour, 1979; Pauly *et al.*, 1992). Accordingly, we could speculate

that the increased plasma and brain KYN, which occurs following chronic nicotine may emanate via the induction of TDO (see: General introduction), derived from a nicotine-stimulated rise in corticosterone concentrations.

The capacity of KYN to move freely between the CNS and periphery is illustrated by the close correlation of KYN values from mouse plasma and whole brain samples. For many of the other kynurenine pathway substances (with the exception of TRP), their ability to traverse the blood-brain barrier is more restricted (Fukui *et al.*, 1991), and like 5-HT the brain levels of these substances may reflect a more regionally selective distribution. Therefore, future experiments may best be conducted by measuring the content of these kynurenines in specific brain areas. Once again, the ability to quantify QUIN could prove highly advantageous.

We have already mentioned how some of the results of these experiments (particularly the chronic nicotine data) appear to contrast with those of certain human studies, for example the biochemical data relating to the TS smokers and non-smokers of our own cross-sectional study (chapter 2). Notable differences, however, exist between these human and animal investigations, which could be used to justify these conflicting findings. Firstly, the doses of nicotine administered to our mice would have produced far higher blood and brain levels than would be achieved from tobacco smoking in humans. For example, in one report the daily intake for a 70kg man who smokes 20 cigarettes a day was estimated as being around 0.4mg/kg (Federal Trade Commission Report, 1971). Alternatively, Benowitz and Jacob III (1984) assessed the daily nicotine intake of 22 human subjects who were habitual smokers averaging one packet per day of medium to high-nicotine yield cigarettes. They calculated the mean daily intake of nicotine as being 37.6mg (± 17.6, Standard Deviation) in their group of smokers. Although Benowitz and Jacob III did not provide details of patients weights, if we calculate the intake based on an assumed 70kg mass, the daily intake of nicotine is approximately 0.5mg/kg. In contrast, for the majority of our experiments the mice received a nicotine dose of 1.6mg/kg (twice daily for chronic nicotine experiments), which represents a much greater intake of nicotine (per unit body weight) over the 24 hour period. Moreover, the levels of nicotine derived from tobacco smoking throughout the day are likely to fluctuate

considerably less, compared with the twice daily injections of nicotine. We should also remember that these animal investigations were essentially designed to provide an insight into how, and indeed whether, nicotine was capable of influencing 5-HT_{2A}-mediated shaking behaviours and/or kynurenine pathway substances in this animal model. Clearly then, it would be interesting to extend these investigations further, using a method of nicotine delivery which could produce more constant body levels of nicotine in the animals throughout a 24 hour period, and at blood concentrations more closely matched to those of human smokers. This could be achieved via slow continuous infusion of nicotine. Furthermore, since the smoking TS patients involved in the large cross-sectional study of Chapter 2, are likely to have smoked over a period of years rather than days, it might be appropriate to consider looking at these nicotine effects over longer time periods as well.

Although we have discussed the possible effects of nicotine in much detail, several experiments have identified pharmacologically active breakdown products of nicotine, and so the potential of these substances to contribute nicotine's neurochemical and behavioural effects should not be ignored. For example, nornicotine, a major alkaloid of tobacco, is a pharmacologically active metabolite of nicotine, which can inhibit high-affinity binding of [^{3}H](−)-nicotine and [^{3}H]-acetylcholine to rat brains (Reavill *et al.*, 1988; Copeland *et al.*, 1991). Despite the [^{3}H](−)-nicotine binding site exhibiting stereoselectivity, as indicated by the 13-fold greater potency of (−)-nicotine compared with its (+)-isomer, there is no clear stereodiscrimination between the (+)- and (−)-nornicotine stereoisomers, both of which have IC_{50} values comparable with (+)-nicotine (Reavill *et al.*, 1988). In a series of drug discrimination and schedule-controlled responding experiments, the two isomers of nornicotine displayed similar profiles of effects when compared with (+)- and (−)-nicotine, differing essentially only in terms of potency (Goldberg *et al.*, 1989). The efficacy of the compounds in the drug discrimination procedure were highly correlated with their potencies for inhibiting binding of tritiated nicotine to rat brain membranes. Contrary to the similarities shown by these tobacco substances in operant behavioural studies, no such correlation was found with cotinine (the major metabolite of nicotine), and its effects were not attenuated by mecamylamine

(Goldberg *et al.*, 1989). Consequently, Goldberg and colleagues proposed that any behavioural effects of cotinine are mediated predominantly via non-nicotinic mechanisms. Moreover, increased serotonin turnover has been observed in the rat brain following peripheral cotinine administration (Essman, 1973), which suggests that cotinine may produce its pharmacological effects via a modulation of serotonergic neurotransmission. In addition, studies indicate that cotinine is able to traverse the blood brain barrier (Crooks *et al.*, 1997), so that any formed as a result of peripheral biotransformation of nicotine could provide a potential source for the brain. However, Crooks and coworkers (1997) were unable to ascertain whether *in situ* conversion of nicotine to cotinine occurs within the brain. (–)-Nornicotine-evoked release of [^{3}H]dopamine from striatal slices has also been demonstrated (Crooks *et al.*, 1997), which was inhibited by the nicotinic antagonists mecamylamine and dihydro-β-erythroidine. On the other hand, norcotinine (another nicotine metabolite) showed no apparent effect on [^{3}H]dopamine release by striatal slices or its uptake into rat striatal synaptosomes (Crooks *et al.*, 1997). Nevertheless, there is substantial evidence to support the possible interaction of these compounds with the biochemical and behavioural actions of nicotine.

The results from this chapter are supportive of an interaction between nicotine and tryptophan metabolic pathways, although the mechanisms involved are unclear. There are several opportunities via which these relationships could be explored further, the results of which could help dictate the direction of future investigations into tic-like behaviours, both in animal and human studies. This could ultimately lead to more acceptable drug strategies for treatment of tic-related conditions.

Table 4.1. Concentration of KYN in plasma and whole brains of mice following acute saline or nicotine (1.6 mg/kg s.c.) treatment, as measured by HPLC.

(10 mins drug pretreatment time; both groups, n=6).

Treatment	Plasma KYN (μmol/L)	Brain KYN (nmol/g)
Saline	1.23 ± 0.23	0.341 ± 0.102
Nicotine (1.6 mg/kg)	1.67 ± 0.36	0.423 ± 0.068

Table 4.2 Whole brain (molar content per gramme of wet tissue) 5-HT and 5-HIAA concentrations, 5-HT turnover, as well as plasma and brain KYN levels measured by HPLC, from mice receiving either single dose or 7 days saline or nicotine (1.6 mg/kg s.c.) treatment.

*, P<0.05 significant difference cf. all other treatments (Duncan's test). All groups, n=6.

Treatment	Plasma KYN (μmol/L)	Brain KYN (nmol/g)	Brain 5-HT (nmol/g)	Brain 5-HIAA (nmol/g)	Brain 5-HIAA/5-HT
Single Saline Dose	0.722 ± 0.091	0.176 ± 0.015	3.592 ± 0.248	2.283 ± 0.067	0.656 ± 0.059
Single Nicotine Dose	0.752 ± 0.072	0.209 ± 0.026	3.468 ± 0.068	2.262 ± 0.072	0.654 ± 0.028
7 Days Saline Dosing	0.803 ± 0.089	0.197 ± 0.011	3.692 ± 0.262	2.353 ± 0.107	0.654 ± 0.057
7 Days Nicotine Dosing	1.058 ± 0.069*	0.300 ± 0.035*	3.370 ± 0.134	2.347 ± 0.092	0.702 ± 0.041

Table 4.3 Concentrations of kynurenine pathway metabolites (as well as KYN:TRP ratio) in mouse plasma, following chronic (7 days) treatment with saline or nicotine (1.6 mg/kg s.c.) as described in methods (and measured by HPLC).

*, P<0.05 significant difference cf. saline control group; Student's t-test (n=6).

Treatment	TRP (μmol/L)	KYN (μmol/L)	KYN/TRP Ratio	KYNA (nmol/L)	HKY (nmol/L)	HAA (nmol/L)
Saline	58.0 ± 4.2	1.26 ± 0.18	0.0215 ± 0.002	52.1 ± 9.2	70.5 ± 10.6	39.4 ± 6.8
Nicotine	70.5 ± 3.9	1.80 ± 0.11*	0.0260 ± 0.003	72.7 ± 5.8	70.0 ± 10.1	37.9 ± 6.6

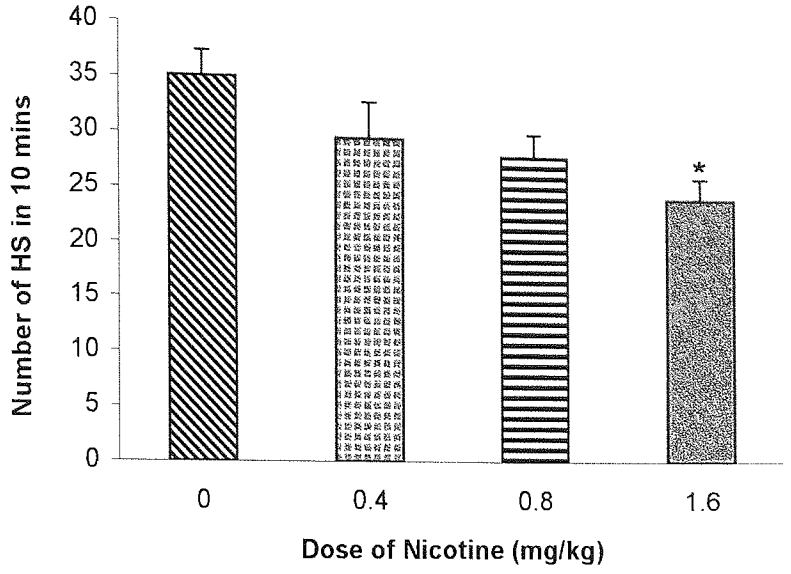

Figure 4.1. The effect of acute nicotine treatment (10 mins. pretreatment time) on DOI-induced HS frequency in mice.

*, $p<0.05$ significant difference cf. mice treated with saline (Duncan's test)(All groups n=6).

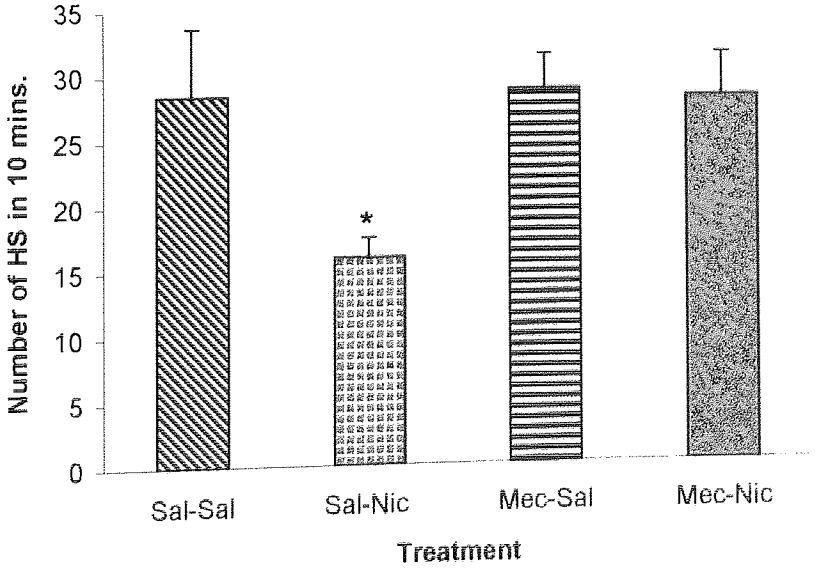

Figure 4.2. The blockade of acute nicotine (1.6mg/kg) effects on DOI-induced HS in mice by mecamylamine (1mg/kg).

*, $p<0.05$ significant difference cf. all other groups (Duncan's test)(All groups n=8).
Abbreviations: Nic=nicotine, Mec=mecamylamine, Sal=saline.

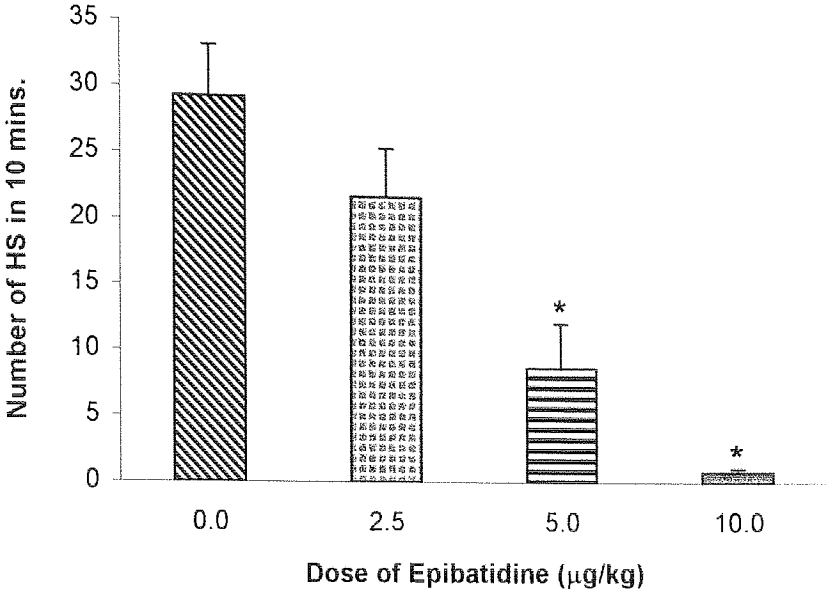

Figure 4.3. The effect of acute epibatidine treatment (10 mins. pretreatment time) on DOI-induced HS frequency in mice.

*, $p<0.05$ significant difference cf. with saline control and mice receiving 2.5μg/kg epibatidine (Duncan's test)(All groups n=6).

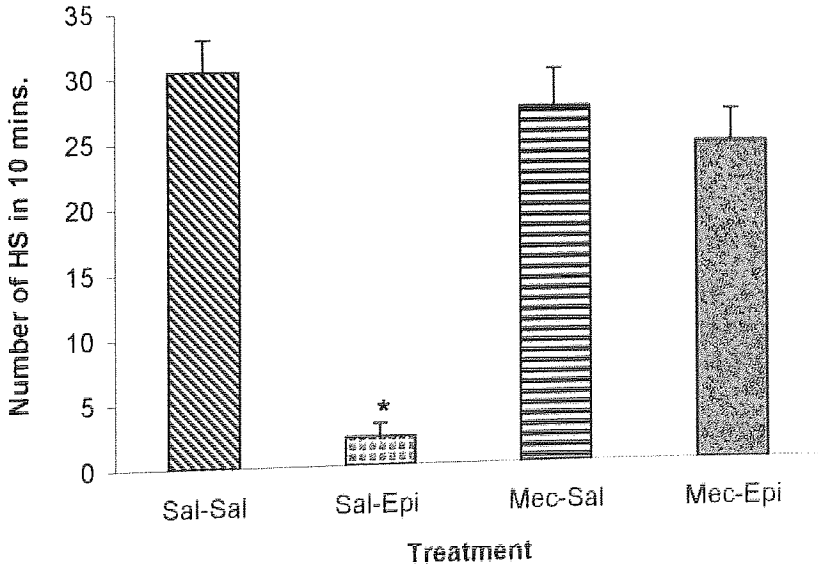

Figure 4.4. The blockade of acute epibatidine (10μg/kg) effects on DOI-induced HS in mice by mecamylamine (1mg/kg).

*, $p<0.05$ significant difference cf. all other groups (Duncan's test)(All groups n=6).
Abbreviations: Epi=epibatidine, Mec=mecamylamine, Sal=saline.

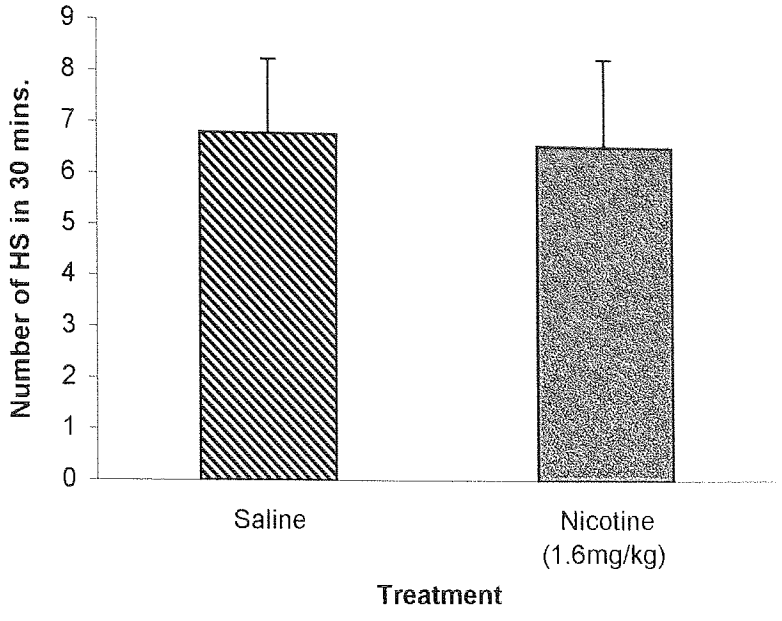

Figure 4.5. The effect of acute nicotine treatment (10 mins. pretreatment time) on spontaneous HS frequency in mice (n=6).

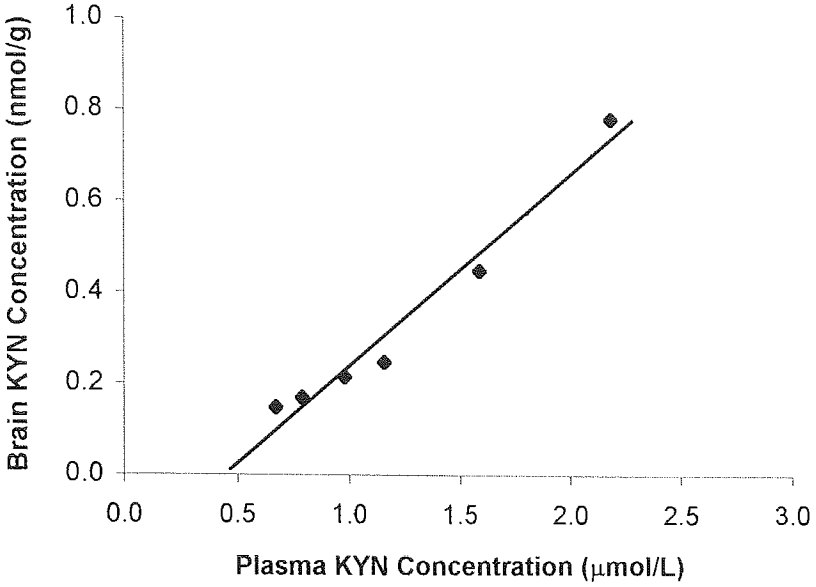

Figure 4.6a. Graph to show correlation between plasma and brain KYN levels after acute saline treatment (10 mins pretreatment time)(n=6).

(r=0.983, p<0.001; Pearson product-moment correlation coefficient).

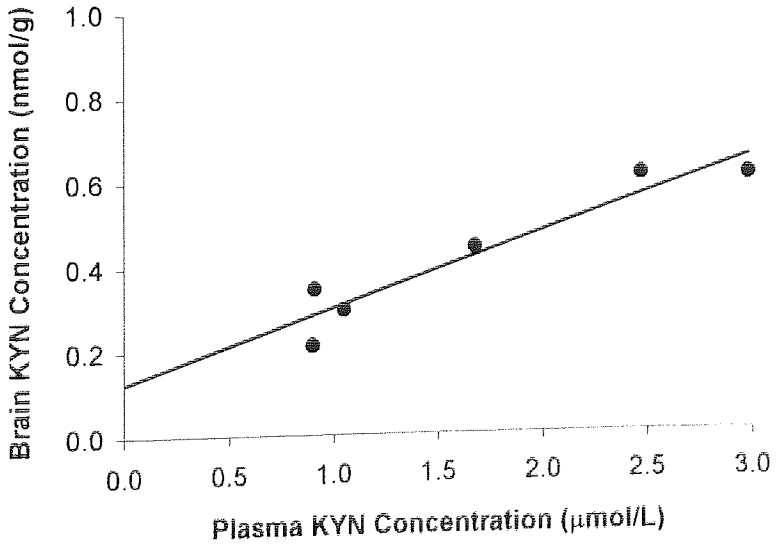

Figure 4.6b. Graph to show correlation between plasma and brain KYN levels after acute nicotine treatment (10 mins pretreatment time)(n=6).

(r=0.950, p<0.001; Pearson product-moment correlation coefficient).

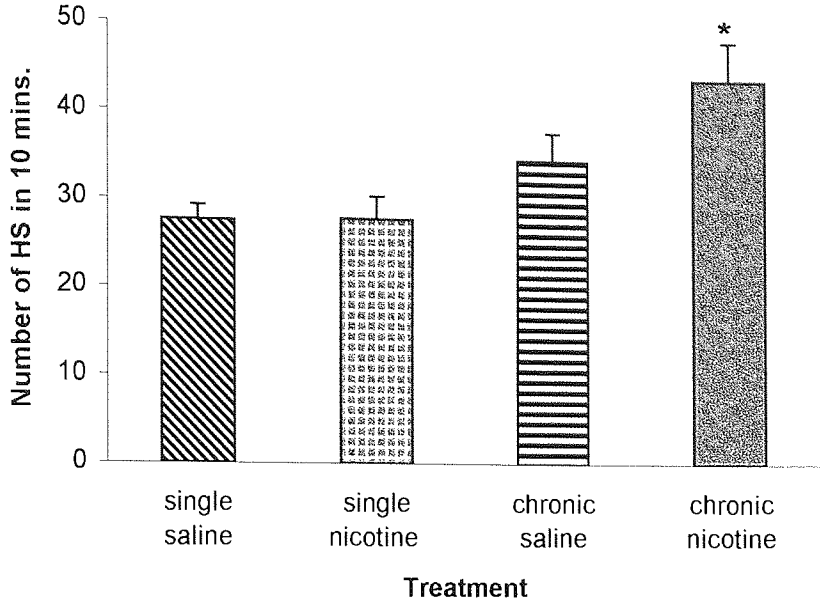

Figure 4.7. The effects of single dose and chronic nicotine (1.6mg/kg) treatment on DOI-induced HS frequency in mice.

*, $p<0.05$ significant difference cf. all other groups (Duncan's test)(All groups n=6).

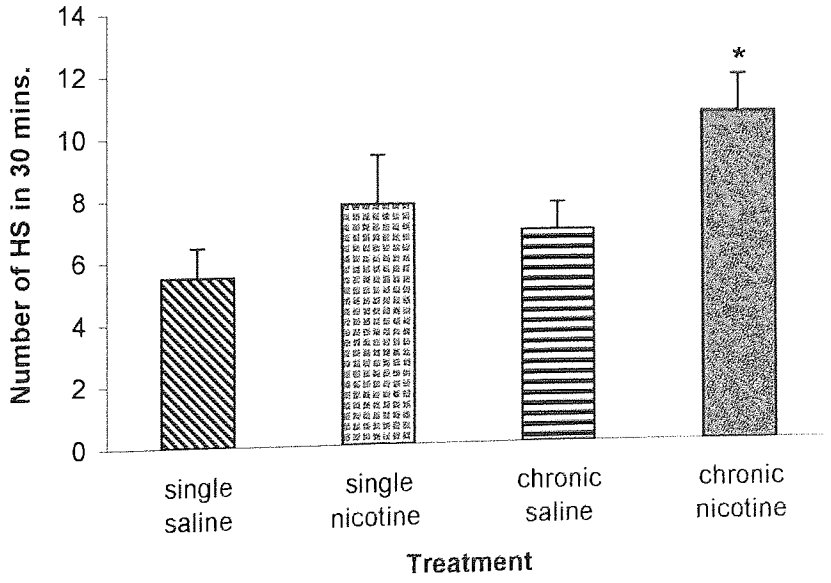

Figure 4.8. The effects of single dose and chronic nicotine (1.6mg/kg) treatment on spontaneous HS frequency in mice.

*, $p<0.05$ significant difference cf. single saline and chronic saline groups (Duncan's test)(for single saline and nicotine groups, n=6; for chronic saline and nicotine groups, n=12).

GENERAL DISCUSSION

It should be evident from the 'General Methods' section that a considerable length of time was invested in the early parts of this PhD toward HPLC assay development, which would allow measurement of kynurenine pathway substances in plasma. Three separate HPLC procedures were subsequently established, owing primarily to the high degree of sensitivity necessary, and this enabled TRP, KYN, KYNA, HKY and HAA to be measured. On the other hand, despite all our attempts to detect and quantify XANA and AA in biological fluids, we were unsuccessful. In addition, in light of its documented neurotoxic properties and a proposed association with various neurodegenerative conditions (see: General Introduction), QUIN provided a further source of disappointment. However, because QUIN is generally quantified using gas chromatography-mass spectrometry (which we did not have access to), our inability to measure plasma QUIN came as little surprise to us.

The results from the initial pilot study (Chapter 1), failed to indicate that diet had any marked effect on the kynurenine pathway variables under consideration. This had a significant bearing on the design of the much larger cross-sectional study (Chapter 2), as well as our longitudinal investigation (Chapters 3), in terms of what dietary restrictions were imposed. However, since the sample size of the TS patient and control groups for controlled feeding and free-feeding were small, this meant that only substantial changes in the biochemical variables were likely to be detected. It is also noteworthy, that the free-feeding group were instructed to eat normally, and were not provided with foods which were either specially modified to lack or contain individual dietary components (for example via TRP supplements). The relevance of this point relates to other studies which have examined the effects of dietary manipulation on TRP metabolic pathways or TS (Ashley *et al.*, 1982; Rasmusson *et al.*, 1997). For example, in the study carried out by Rasmusson *et al.* (1997), in which he investigated the effects of TRP depletion on tic, obsessive-compulsive, or mood symptoms in TS, TS subjects were provided with a diet containing low levels of TRP, or alternatively were given TRP capsules (containing 500mg TRP) with their meals. In contrast to the objectives of these studies, our aim was to try to establish a

protocol for the cross-sectional study where dietary factors remained stable in all cases. This could allow us to assume that any changes in the kynurenine pathway variables between TS subjects and controls, were probably linked in some way to the presence of TS in affected individuals, rather than merely a result of variations in their diets. For the same reasons (*i.e.* their possible influence on TRP and its metabolism), caffeine, aspirin and nicotine intake was prohibited on the day of blood sampling (refer to: Methods section of chapters 1 and 2). In the large cross-sectional study, an effect of diet on TRP was picked up, although no effect was detected for the other biochemical variables.

The data from the pilot and cross-sectional investigations respectively, demonstrated some influence by diurnal and seasonal factors on the biochemical variables. This is most clearly reflected in the cortisol measurements, where early morning peaks (see: chapter 1) and a seasonal fluctuation (see: Chapter 2) were observed. It is thus important that their potential effects on other substances, either directly or indirectly, are not underestimated, particularly with regard to the design of future human or animal investigations. Accordingly, it is well documented that TS symptoms exhibit a waxing and waning nature, and one could speculate that they too may reflect a seasonal fluctuation within individuals. Unfortunately, owing to the short time over which the longitudinal study was conducted, and the relative paucity of data produced (refer to: Chapter 3, Discussion), we were unable to carry out an adequate examination of this hypothesis. Moreover, it was difficult to statistically prove that any alterations in TS symptom severity were associated with changes in the kynurenine pathway variables. However, while on the one hand there are obvious benefits to be gained from lengthening the course of future studies concerned with changes in TS symptom severity, we found to our cost that guaranteeing patient reliability (*i.e.* for keeping all of their agreed appointments) is a huge consideration, and a potential source of extreme frustration for the researcher.

One of the prime objectives of this thesis was to establish the reported increase in plasma KYN in TS (Dursun *et al.*, 1994a). No difference in KYN was found in the small pilot study (Chapter 1), but KYN was significantly elevated in our larger cross-sectional investigation (Chapter 2), although the magnitude of the increase was

somewhat smaller than that detected by Dursun and colleagues (1994a). We propose that procedural differences and seasonal factors are the most likely source of these inconsistencies (refer to discussions in Chapters 1 and 2). We further hypothesise that although TS is frequently characterised by a general increase in KYN for a given sample population of TS patients, the rise in KYN is not reflected in all affected subjects, as was originally thought by Dursun *et al.* (1994a). Indeed, since the spectrum and severity of TS symptoms (as well as associated features) can vary markedly between individuals, it may be that the biochemical picture will differ significantly between cases too. Neopterin differences in TS acted as a further stimulus for discussion, since Dursun *et al.* (1994a) found no change in plasma neopterin. In contrast, neopterin was significantly raised in the much larger TS sample population of our cross-sectional study, thereby indicating that this increased KYN in TS most likely emanates from an induction of IDO (refer to: General Introduction). Moreover, since IDO and GTP-cyclohydrolase I (the first enzyme in the formation of neopterin) are strongly induced by cytokines, the simultaneous increase in KYN and neopterin is suggestive that an increased flux of TRP through the kynurenine pathway might be occurring through a process related to cellular immunity, in some parts of the body or brain. What happens to the increased KYN, however, was not clear because neither KYNA, HKY or HAA were altered in the plasma of TS patients. This result serves to emphasise the potential advantages of being able to detect QUIN in biological fluids, since accumulation of QUIN has previously been found to correlate closely with stimulation of the immune system within the CNS and increases in IDO activity (which also resulted in elevated KYN) in animal models of inflammatory disease (Heyes *et al.*, 1992b), as well as patients with CNS infections or autoimmune conditions (Heyes *et al.*, 1992a, Heyes *et al.*, 1995). Nevertheless, since these kynurenines have a restricted access between the central and peripheral nervous systems (Fukui *et al.*, 1991), it is possible that any changes that do occur are restricted to localised regions of the brain. The benefits of finding a means of measuring QUIN would undoubtedly provide huge scope for additional research into tic disorders, through both animal and clinical investigations. Consequently, one of the most obvious next steps for human clinical research would

surely involve a biochemical comparison of samples collected from the CNS (*i.e.* CSF fluid) of TS subjects and matched controls. In addition, it would be beneficial to carry out measurements of cytokines in future studies, which could provide further information of the importance of the immune system to tic disorders.

There have been two previous reports of reduced plasma TRP in TS (Leckman *et al.*, 1984, Comings, 1990a), although Dursun and colleagues (1994a) found no change in the plasma levels of this amino acid. In our pilot study, we observed a trend towards decreased TRP in TS patients, but the difference was not significant. In the cross-sectional study there was no evidence of altered TRP in TS. One factor which may be important to these conflicting results could be time of day. In both of the studies where a difference in TRP was reported, blood samples were collected early in the morning, and the trend towards reduced TRP in TS patients (in the pilot study) related to samples obtained at 09:30 hrs. In contrast, many of the samples from the large cross-sectional investigation were collected in the afternoon, due to various reasons, such as the considerable distances that our TS subjects had to travel to the clinics. Nevertheless, potential diurnal influences of are once again highlighted.

Although no change in TRP was found between TS subjects and controls in either of the relevant investigations (Chapters 1 and 2) within this thesis, TS subjects with a family history of tics had lower TRP than those subjects with no known family history (see: Chapter 2), and this difference could provide a basis for further research. The well documented hereditary nature of TS (see: General Introduction), leads us to speculate on the possible association between certain biochemical variables in samples obtained from TS subjects and their immediate relatives.

Animal studies have shown that systemic KYN pretreatment potentiates both 5-HTP- and 5-HT-induced HS (Handley and Miskin, 1977), as well as DOI-HS (McCreary and Handley, 1995), suggesting that KYN does play a role in the mediation of tic-like behaviours. On the other hand, ligand binding studies show that KYN, and other kynurenine pathway substances, have no appreciable affinity for 5-HT_{2A} receptors (via which these animal shaking behaviours are predominantly mediated), or for 5-HT_{1A} receptors (Kariyawasam *et al.*, 1997), which functionally interacts with the 5-HT_{2A} receptor subtype (Dursun and Handley, 1993; Schreiber *et al.*, 1995). The

freedom with which KYN travels between the CNS and periphery is well documented (Gal and Sherman, 1980), and the close correlation between plasma and brain KYN from our animal data is certainly supportive of this widely accepted concept. While there is plenty of evidence to show that KYN can modulate 5-HT_{2A}-induced shaking behaviours, the mechanism of this effect is less clear. Handley and Miskin (1977) showed that low doses of HKY produced a similar potentiation of 5-HT and 5-HTP-induced HS in mice, but the effects of other KYN metabolites (particularly QUIN) on animal shaking behaviours remains a potential source for further investigation. However, given their poor penetration of the CNS, this would necessitate that they be injected directly into the brain.

The decrease in the plasma KYN and KYNA of TS smokers compared with TS non-smokers begs the question of where the interaction between the kynurenine pathway and nicotinic cholinergic system lies, and of course its significance to tic conditions. These results served as a precursor to the animal studies of this thesis, which not only supported a likely association between the systems, but provided scope for further research. Our investigation of the effects of nicotine on shaking behaviours in mice (see Chapter 4) indicated that acute nicotine treatment resulted in a dose dependent inhibition of DOI-induced HS (but not spontaneous HS), and that the effect was mediated via nicotinic cholinergic receptors. In contrast, twice daily nicotine treatment for a period of 7 days caused an increase in both spontaneous and 5-HT_{2A}-induced HS, although the mechanism of the effect was unclear. Plasma and whole brain KYN concentrations were increased following 7 days nicotine treatment, but no difference was found after acute nicotine. The apparent incongruity between the effects of nicotine in the human (see: Chapter 3) and animal studies (Chapter 4), makes it imperative to recognise the existence of fundamental differences in the experiments (see: Chapter 4 discussion). Therefore, a direct comparison of the data is wholly inappropriate. Given the relatively brief period over which the mice received nicotine (7days for chronic experiments), the small number of daily doses per animal, and the higher nicotine intake by the mice compared with cigarette smoking in humans, adjustments to the design of future studies are necessary. For example, use of slow continuous infusion could enable a more constant delivery of nicotine, and

experiments could be lengthened to look at the longer term effects of nicotine exposure. It is also noteworthy that clinical studies relating to nicotine's efficacy in TS treatment (see: General Introduction) have not looked at its effects on symptoms over the longer term.

Analysis of whole brains from mice treated with nicotine (over 7 days) failed to indicate an alteration in either 5-HT or 5-HIAA concentrations, or 5-HT turnover. However, regional decreases in 5-HT and 5-HIAA (particularly in the hippocampus) in nicotine treated rats has been demostrated (Benwell and Balfour, 1979). Moreover, studies indicate that the rate of L-tryptophan uptake by hippocampal synaptosomes is reduced in rats injected chronically with nicotine, via a decrease in the number of amino acid carrier molecules in the nerve terminal membrane (Benwell and Balfour, 1982). Such a mechanism allows us to speculate about the possiblity of a regional decrease in 5-HT activity in rats receiving chronic nicotine, which could induce changes in shaking behaviour. On the other hand, one must accept that a global reduction in 5-HT turnover cannot itself account for the precipitation of tics, because tics are not a feature of other disease states characterised by reduced 5-HT turnover. For example, decreases in 5-HT turnover via a low TRP diet does not result in tics (Young *et al.*, 1985), or indeed lead to a worsening of tics (Rasmusson *et al.*, 1997). Furthermore, some depressed patients have exhibited severe reductions in CSF 5-HIAA without tics being reported (Asberg *et al.*, 1976). Alternatively, there are no reports of procedures which deplete 5-HT having produced tic-like movements in animals. Nonetheless, a reduction in 5-HT function can supress head twitches. For instance, 5-HT_{1A} receptor agonists (which inhibit raphe firing) invariably act as inhibitors of 5-HTP and DOI-induced HS (Goodwin and Green, 1985; Dursun and Handley, 1993; Schreiber *et al.*, 1995). In contrast to these potential interactions between nicotinic cholinergic and serotonergic mechanisms, Shytle et al. (1995) found that nicotine attenuates kainic acid-induced WDS in rats, and theorised that these effects could emanate via nicotine interactions with glutamate receptors or via GABA-mediated processes (see: Chapter 4 discussion). The well known association of KYNA and QUIN with the NMDA class of excitatory amino acid receptors invites further reseach into the proposed association of these neurotransmitter systems, and their relevance to tic-like behaviours. Consequently,

although serotonergic pathways may well figure in the equation of the underlying pharmacology for tic symptom expression in humans and in relevant animal models, the whole picture is obviously far more complicated, and most likely involves several different neurotransmitter systems.

It has been hypothesised that there is an association between prior streptococcal infection (specifically β-haemolytic streptococcal infection), and the onset or worsening of certain neuropsychiatric conditions, including TS (Kiessling *et al.*, 1993; refer also to General Introduction) The data from Chapter 2 did not indicate any difference in the number of TS patients with positive ASO titres compared with the controls, but since the TS patients recruited for our study were not necessarily recently diagnosed cases of TS, this outcome could only been expected. In addition, no obvious correlation between TS symptom severity and the frequency of positive ASO titre results was exhibited in the cross-sectional (Chapter 2) or longitudinal (Chapter 3) investigations, although as we have already discussed the lack of data from the latter study meant that a detailed statistical analyses was not possible. Previous reports, which support an association between the precipitation or exacerbation of tics and infection from β-haemolytic streptococci indicate that the time when samples are collected is a critical factor, and this must be borne in mind for any future investigations of this issue. Preliminary studies within this research group did not detect any increase in spontaneous or DOI-induced HS after injecting mice with dead streptococci for 3 weeks (see: Chapter 3; discussion), but it might be worthwhile to utilise this model (after suitable modifications) to continue to explore the hypothesis.

Another possible interesting source of discussion related to the decrease in plasma KYN, TRP and KYNA of clonidine treated TS patients compared with unmedicated TS subjects. Replication of this result is surely warranted before we can confidently predict a clonidine influence on the activity of the kynurenine pathway. Nevertheless, the opportunity exists for investigating the basis of this effect and its possible relevance to the efficacy of the drug in tic disorders. In addition, we could ask why

other medications equally or more effective than clonidine in treating TS symptoms (such as haloperidol) failed to alter these kynurenine pathway substances. Perhaps a useful place to begin would be to assess what effect acute and chronic clonidine injections to mice might have on the levels of substances in plasma and brain samples. Measuring spontaneous HS in these clonidine treated mice would help to equate any changes in biochemistry with the effects of the drug on shaking behaviours.

In conclusion, the hypothesis of increased KYN in TS appears to be confirmed by these studies. The corresponding increase in neopterin in TS suggests that the source of abnormal KYN metabolism most likely originates from induction of IDO, which indicates a possible autoimmune basis for the disorder. However, it is not entirely clear whether these changes in KYN and neopterin are fundamental to the expression of the disease state, or merely a secondary effect of some other underlying biochemical abnormality. The significantly higher plasma KYN in non-smoking TS patients compared with TS smokers, and the ability of acute and chronic nicotine treatment to modify HS and KYN concentrations in mice, suggest that the nicotinic cholinergic system may have significant role to play in the pathophysiology of TS. The development of more refined assay procedures designed to measure further kynurenine pathway substances, and an extension of the studies carried out to date can only further our understanding of these subjects.

Suggestions for further studies:

1. Studies are indicated to investigate the effects of seasonal and diurnal factors in TS symptoms.
2. An extension of longitudinal studies are indicated to more fully investigate the effects of correlation between TS symptom severity and the frequency of positive ASO titre readings.
3. An assessment of the effects of streptococcal infection in mice on the HS model and on selected biochemical moieties are suggested.
4. Further studies have been suggested to assess the effects of other kynurenine pathway components on DOI-induced (and possibly spontaneous) HS.

5. We propose that a clinical investigations of changes in KYN metabolism between TS patients and matched controls should be expanded to include comparable numbers of smokers and non-smokers within each sample population.

6. The effects of nicotine on rodent HS and tryptophan metabolic pathways should also be extended. This could involve nicotine administration to mice via slow continuous infusion over varying lengths of time, to evaluate the longer term effects of nicotine on shaking behaviour and its effects on peripheral and brain biochemicals.

6. More extensive examination of clonidine effects on kynurenine pathway substances in mice is indicated.

7. Measurement of kynurenine pathway substances in cerebrospinal fluid and plasma from TS subjects and controls is suggested.

8. The development of assays to measure additional KYN metabolites (particularly QUIN) in biological fluids could also benefit future studies.

9. Measurement of cytokines (for example γ-interferon) in TS subjects and controls is also suggested.

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