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SEROTONIN RECEPTOR MECHANISMS IN ANTI- DEPRESSANT ACTION

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' 2C or not 2C, that is the question, whether 'tis nobler in the mind to suffer the slings and arrows of outrageous fortune or ... by opposing, end them.'

Paraphrasing Hamlet in 'Hamlet', Act 3, Scene 1,
by William Shakespeare.

ABSTRACT

Background: Serotonin neurones have been implicated in the pathophysiology and treatment of clinical depression to a greater degree than any other neurotransmitter. Additionally, serotonin pathways may play a role in the pathophysiology and treatment of eating disorders, anxiety states and schizophrenia. Molecular biological studies have confirmed pharmacological evidence suggesting the existence of multiple serotonin receptor subtypes and the genes for these receptors, as well as that of the serotonin transporter, have common polymorphic variants.

Aim: To investigate the effect of repeated treatment with selective serotonin re-uptake inhibitors (SSRI's) on the function of central 5-HT_{2C} receptors. To assess the effect of polymorphic variation in the 5-HT_{2C} receptor and serotonin transporter on functional responses to selective pharmacological challenge. To determine whether polymorphic variation in the 5-HT receptor and serotonin transporter influence the clinical response of patients with major depression to treatment with serotonergic antidepressants.

Methods: To assess the effect of repeated treatment with selective serotonin re-uptake inhibitors (SSRI's) on the function of central 5-HT_{2C} receptors I used the 5-HT_{2C} receptor agonist, m-chlorophenylpiperazine (m-CPP) as a 5-HT_{2C} probe in a neuroendocrine challenge paradigm. I used the same approach to assess whether polymorphic variation in the 5-HT_{2C} receptor (serine vs cysteine substitution) was associated with differences in functional response to 5-HT_{2C} receptor challenge. I then studied whether polymorphic variation in the serotonin transporter promoter region (long versus short form) was associated with differing functional responses to acute challenge with clomipramine, a tricyclic antidepressant with a high affinity for the serotonin transporter. Finally, I studied whether either of these polymorphic variants influenced the clinical response of patients with major depression to treatment with SSRI's and clomipramine.

Results: SSRI treatment significantly lowered the sensitivity of 5-HT_{2C} receptors as predicted from animal experimental studies. However polymorphic variation in the 5-HT_{2C} receptor did not significantly influence functional responses to m-CPP challenge. In contrast polymorphic variation in the serotonin transporter was associated with differing neuroendocrine responses to acute clomipramine challenge with greater prolactin release being seen in subjects with the long polymorphic variant. Neither the 5-HT_{2C} nor the transporter polymorphisms correlated with clinical response to SSRI and clomipramine treatment in patients with major depression.

Conclusions: The ability of SSRI's to produce a functional down-regulation of 5-HT_{2C} receptors may be relevant to certain of their therapeutic effects. Polymorphic variation in the 5-HT_{2C} receptor (serine vs cysteine) seems unlikely to explain functional differences in responses to 5-HT_{2C} receptor challenge or antidepressant responses to SSRI treatment. In contrast variation in the serotonin transporter promoter is associated with differing functional responses to acute serotonin re-uptake blockade. However, this did not correlate with clinical response to longer-term SSRI treatment.

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ABBREVIATIONS

ACCh:	Acetyl Choline
ACTH:	Adrenocorticotrophic Hormone
ANOVA:	Analysis of variance
AUC:	Area under the curve
AUC-B:	Area under the curve minus baseline
BDI:	Beck Depression Inventory
BDNF:	Brain derived neurotrophic factor
bp:	Base pair
BPRS:	Brief Psychiatric Rating Scale
5-HT:	Serotonin
c-AMP:	Cyclic Adenosine Mono-phosphate
CCK:	Cholecystekinin
cDNA:	complementary De-oxyribo Nucleic Acid
CGI:	Clinical Global Impression
CIT:	Citalopram
CSF:	Cerebro-spinal Fluid
CPM:	Cycles per minute
CV:	Co-efficient of variation
DMSO:	Di-Methyl Sulph-oxide
DNA:	De-oxyribonucleic acid.
DOI:	1-(2.5-dimethoxy-4-iodophenyl)-2-aminopropane
DRN:	Dorsal raphe nucleus
DSM III R:	Diagnostic and Statistical manual of mental disorder (Third edition - revised)
DSM IV:	Diagnostic and Statistical manual of mental disorder (Fourth edition)
ECT:	Electro-convulsive treatment
GABA:	Gamma amino-butyric acid
GH:	Growth hormone
GIT:	Gastro-intestinal tract
HAM-D:	Hamilton Rating Scale for Depression
HPLC:	High Performance Liquid Chromatography
HVA:	Homo Vanillic Acid
IV:	Intra-venous
5-CT:	5-carboxyamidotryptamine
5-HTTLPR:	Serotonin Transporter Promoter Region
5- HIAA:	5-Hydroxyindoleacetic acid
5-HT:	5-Hydroxytryptamine
5-HTT:	Serotonin Transporter
8-OH-DPAT:	8-Hydroxy-2-(Di-n-propylamino)-Tetralin
ICD 10:	International Classification of Diseases – tenth edition
ICV:	Intra-cerebral ventricles
IPSP:	Inhibitory Post-synaptic Potentials

kJ:	Kilojoules
LSH:	Lutein Stimulating Hormone
MADRS:	Montgomery Asberg Depression Rating Scale
MAO:	Monoamine oxidase
MAOI:	Monoamine oxidase inhibitors
<i>m</i> -CPP:	Meta-chlorophenylpiperazine
mIU/l:	Milli International Units per litre
MHPG:	3 methoxy-4-hydroxyphenyl glycol
MRC:	Medical Research Council
m-RNA:	Messenger RNA
MRN:	Median raphe nucleus
NA:	Noradrenaline
NARI:	Noradrenaline Re-uptake Inhibitor
NASSA:	Noradrenaline and serotonin specific anti-depressant
NS:	Non-significant
NSB:	Non-specific binding
OCD:	Obsessive Compulsive Disorder
OPREC:	Oxfordshire Psychiatric Research Ethics Committee
PCR:	Polymerase Chain Reaction
PRL:	Prolactin
PVN:	Paraventricular nucleus
REM:	Rapid eye movement
RIMA:	Reversible Inhibitor of Mono-amine oxidase
RNA:	Ribo-nucleic acid
RPM:	Revolutions per minute
SCID:	Structured Clinical Interview for DSM4
SPE:	Solid Phase Extraction
SSRI:	Serotonin selective re-uptake inhibitors
SNRI:	Serotonin and noradrenaline re-uptake inhibitor
TCA:	Tricyclic antidepressant
TDT:	Transmission disequilibrium
TFMPP:	1-(3-(trifluoromethyl)-Phenyl) Piperazine
TMS:	Transcranial Magnetic Stimulation
TRH:	Thyroid releasing hormone
TRP:	Tryptophan
UV/VIS:	Ultra-violet light visible
VAS:	Visual analogue scales
VNTR:	Variable Number of Tandem Repeats

DECLARATION

I, Digby John Quested, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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CHAPTER 1

5-HYDROXYTRYPTAMINE AND PSYCHIATRIC DISORDERS

1.1 INTRODUCTION TO THE THESIS

This thesis investigates the function of two molecules involved in the serotonin neurotransmitter system, the 5-HT_{2C} receptor and the serotonin transporter. Interest in these receptors derives from the well established link between the serotonin system and depression and, more recently, a putative link between the response to the new anti-psychotic medication in patients with schizophrenia and 5-HT_{2C} (and other serotonin receptor) blockade.

Knowing the effect of serotonergic medication on specific receptor activity is important in the attempt to develop a full understanding of the mechanism of action of the anti-depressants. It is hoped that a fuller understanding of these mechanisms will facilitate the development of more effective treatments, both in terms of rates of response and also speed of onset of action. The introductory chapter (Chapter 1) and the methods chapter (Chapter 2) are therefore followed by the first experimental chapter which deals with the effect of three weeks of paroxetine treatment on the level of function of the 5-HT_{2C} receptors, using the relatively specific agonist, m-CPP (Chapter 3).

The introductory chapter presents the background to the clinical condition of depression as well as summarizing current views on the aetiology and treatment, both biological and psychological. The serotonin system is reviewed and the current level of knowledge regarding the function of the different receptors is noted. Attention is then focused on the receptors of specific interest, initially from a historic and functional perspective and later with reference to molecular aspects.

It was also hoped, in planning these experiments, that the relatively new insights from the field of molecular biology might provide a further means of differentiating those patients likely to respond to specific drug treatments from those likely to be resistant to the effects of certain classes of drug. In Chapter 4, therefore, a sample of depressed patients was genotyped for polymorphisms of the above two receptors and response rates were analysed by using a global clinical improvement scale. The rates of the polymorphisms were also compared with those in a large control sample without previous or current affective disorder.

In Chapter 5 m-CPP was again used as a probe to investigate possible differences between the function of the two alleles of one 5-HT_{2C} polymorphism (serine substitution for cysteine at codon 23), as a correlation of the serine allele with clozapine response in schizophrenic patients had suggested that functional differences were likely unless linkage disequilibrium was occurring. In a similar manner, in Chapter 6 possible functional differences in the alleles of the serotonin transporter promoter polymorphism were investigated using clomipramine as the neuro-endocrine probe.

The overall findings are further discussed in Chapter 7 and possible lines of further research, based on the implications of these projects and other lines of enquiry, are considered.

1.2 DEPRESSION - INTRODUCTION

Depression is one of the earliest recorded types of mental illness having been mentioned in the writings of Hippocrates, approximately 500 years BC (Jackson, 1986). At that time all illness was divided into representations of an excess or deficiency of “bile” types. Depression was therefore associated with “black bile”.

Literature has recorded depression in both non-medical and medical contexts for as long as we have records of writings from history. In 1621, Robert Burton, from Oxford, who was not medically trained, wrote about melancholia and is thought to have first conceptualised the ‘modern medical doctrine of depression’ (Shorter, 2001).

In the early 19th century Pinel, from Paris, predicted modern findings regarding increased familial rates of depression with his concept of ‘melancholic constitution’ (Pinel, 1809). The recent clinical history of depression has seen different phases of conceptualisation of the illness.

Although early papers argued the likely role of psychological aetiological mechanisms, such as “Mourning and Melancholia” by Sigmund Freud (Freud, 1917), subsequent models of the illness have taken into account both biological and psycho-social factors.

The psychodynamic formulation of depression proposed an ‘unconscious’ loss, of a symbolic nature, although depressive equivalents, such as bereavement, could also result from objective loss in the psychosocial environment. Freud wrote his paper in order to compare and contrast the clinical state of melancholia with the symptom profile of those recently bereaved. It is generally agreed that there are similarities but that in severe depression certain symptoms are more likely to be exaggerated, for example guilt and suicidal thinking. Attention has gradually shifted away from this psychodynamic formulation for a number of reasons. One central problem concerned the validity of Freud’s model of human psychological function which has been increasingly criticised from a number of perspectives. One of these is his notion of the primacy of aberrant sexual development in the formation of pathological states of mind (Freud, 1905). Although subsequent evidence has confirmed his suspicion that child sexual abuse is widespread, later recanted to a description of likely fantasy, it is now recognised that the number of relevant aetiological factors in the development of depression is usually greater than can be explained by a single negative life experience and, in view of current knowledge about genetic mechanisms, it is recognised that some individuals will become depressed without any evidence of major negative life events. An early major breakthrough in the treatment and investigation of the disorder was the discovery in 1958 that iproniazid, a treatment being used for tuberculosis, was also able to elevate the mood of depressed patients.

This discovery, and the knowledge that iproniazid had an influence on mono-amine systems, generated a search for further compounds which would have a similar or improved effect. Therefore, subsequent compounds to be studied included imipramine and other monoamine re-uptake inhibitors.

In view of the success of psychotropic medication in providing sufferers with relief from the symptoms of depression and schizophrenia since the late 1950's, it has become a goal of psychopharmacological research to optimise the efficacy of compounds available in the treatment of severe psychiatric disorders. As far as the treatment of mood disorders is concerned there has been a significant increase in the number of medication types available for this purpose which has led to the development of algorithms facilitating the decisions regarding the choice of medications to be used. At the same time there has been an increase in the numbers of large scale clinical trials to prove the efficacy of the compounds and in order to test different augmentation strategies. Much recent research has been concerned with establishing a role for the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) in the pathophysiology of depression and the therapeutic effects of antidepressant medication. This thesis focuses on the role of the serotonin receptors in the regulation of mood and the manner in which serotonin receptor sensitivity may be influenced by antidepressant and anti-psychotic medication. The work follows from the general hypothesis that a better understanding of the psychopharmacology of psychiatric illness will lead to knowledge about the relevant aetiological factors and, once these are known, further work can be directed at elucidating optimal therapeutic and preventative strategies.

1.2.1 Classification

Along with the differing views of the nature of depression, the classification systems have been modelled on the prevailing concept of the illness at any particular time. Depression was divided historically into “endogenous” and “reactive”. The division was based on the underlying concept that these two kinds of depression differed both in aetiology and in characteristic symptom profile. Although this division had some utility in the planning of medical or psychosocial interventions, it was not consistently established that the two conditions separated well when statistical factor analysis was utilised (Kendell, 1976).

It has also become evident that this dichotomous classification does not have great therapeutic implications as antidepressants have been found to be effective, both in moderate and severe depression as well as in mild or brief recurrent depression (Paykel et al, 1989). Subsequent attempts at classification have been partially useful but usually in the specific context within which they were developed. Paykel proposed a classification (Paykel, 1971) and in the same year, Guze proposed a division into primary and secondary depression depending on the evolution of the condition and the likelihood that it was precipitated by either psychosocial or medical events (Guze et al, 1971), however the validity of this model has not been supported from either a clinical or research perspective (Weismann et al, 1977).

A more useful classification was proposed by Leonhard (Leonhard et al, 1962) who suggested a division of mood disorders into unipolar and bipolar disorder.

This division has been accepted for both its clinical and research utility by the American Psychiatric Association in the Diagnostic and Statistical Manual (Classification of Mental Illness) IV, used widely in both the United States of America and other countries (DSM IV, 1994).

The division of psychotic illness into dementia praecox (schizophrenia) and the mood disorders was described by Kraepelin (1921) and, although the division is not regarded as complete, the recognition of psychotic symptoms as part of a mood disorder has significant therapeutic implications. The DSM IV classification system describes clinical depression as “major depression”. The criteria for diagnosis of this disorder are seen in Table 1.1.

The diagnosis requires a minimum two week period of low mood or loss of interest on most of the days in addition to a further 4 out of 9 other criteria. Bipolar disorder requires both the history of depressive episodes as well as manic or hypomanic episodes (Bipolar I or II respectively) while the ICD 10 classification permits the diagnosis of “manic episode”.

However, the ICD classification system usefully divides the condition into levels of severity - mild, moderate and severe. Mild and moderate depression is further divided according to the presence or absence of somatic symptoms such as an increase or decrease in appetite, weight, sleep, motor activity and energy, and “severe” depression is divided into “with or without psychotic symptoms” (WHO, 1992).

Features include loss of interest or pleasure and a lack of reactivity to pleasureable stimuli, plus three of the following - a variation in the qualitative nature of the mood, early morning waking, psychomotor agitation or retardation, significant anorexia or weight loss, and excessive guilt.

Table 1.1: Criteria for major depressive episode in DSM IV.

A. One of the following two criteria is essential (assuming no other clear cause):	
1	Depressed mood most of the day, nearly every day, as indicated by either subjective report (eg: feels sad or empty) or observation made by others.
2	Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (subjective account or objective view).
B. Three of the following criteria if both above criteria or 4 if one above:	
1.	Increase or decrease in appetite nearly every day, or weight (more than 5% in one month).
2.	Insomnia or hypersomnia nearly every day.
3.	Psychomotor agitation or retardation nearly every day.
4.	Fatigue or loss of energy nearly every day.
5.	Feelings of worthlessness or guilt (may be delusional) nearly every day, not just about being sick.
6.	Diminished ability to think or concentrate, or indecisiveness, nearly every day (either subjectively or as observed by others).
7.	Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a plan or a planned intention to commit suicide.

The division of depression into unipolar and bipolar disorders has been supported in further research relating to the genetic aetiology of the condition as well as with regard to the gender prevalences of the illness. It is therefore likely that the unipolar/bipolar distinction will continue and, from a clinical perspective, the division into mild, moderate and severe has practical utility. With regard to the decision to use electro-convulsive therapy, the presence of delusions and retardation have been ascertained to be important factors (Brandon et al, 1984).

1.2.2 Epidemiology

Depression is a common disorder and lifetime rates are currently reported between 4% and 30%, while point prevalences of between 13 to 20 % of the population have been reported (Reviewed in Joyce, 2000). Increased rates are seen in women, individuals from social classes 4 and 5 and those who are separated or divorced (Boyd and Weismann, 1982).

There has been a steady increase in the estimate of the global prevalence of the disorder from 100 per million in the late 1950's to over a 100 000 per million in the last years of the 20th century (Kessler et al, 1994). In the United Kingdom the National Morbidity Survey indicated that, in any week, 7.7 % of the population suffers from 'mixed anxious depression' and 2.1 % from 'pure depressive' episodes (HMSO, 1995).

A significant number of people are not treated by doctors at all, but it is recognised that most people (90 %) suffering from depression are treated in primary care by their general practitioners, rather than by specialists, (Shepherd et al, 1966) and the precise calculation of incidence and prevalence rates is further complicated according to factors dictating help seeking behaviour.

Research done in community studies recognises that there is a greater prevalence of the illness in women and this has taken into account the likelihood that they present at a lower threshold than men in primary care.

The 2:1 female to male ratio is a consistent finding, with the one exception being an Amish study where the ratio was 1:1 and 49 % of the males had suffered from depression (Egeland et al, 1983), however this is a skewed sample in view of the substantial genetic risk in the Amish men.

In the early epidemiological studies the development of a mood disorder was seen to be more likely with increasing age up to a mean age of onset of 30 years (Smith and Weismann, 1992), however recent studies have suggested an earlier mean of 21 years. Following the initial episode further episodes are likely in the majority of sufferers and these have been documented as occurring more frequently with increasing age (Roy-Byrne et al, 1985).

1.2.3 Aetiology

1.2.3.1 Mono-amine hypothesis

The mono-amine hypothesis (Schildkraut, 1965), that depression relates to a functional deficit of mono-amine transmitters, while the reverse is true in mania, derives from a number of related findings. It was recognised at an early stage that treatment of hypertension with reserpine and guanethidine, while being effectively anti-hypertensive, led to depression in a proportion of people taking the medication. It had been previously established that these medications function by depleting neurones of their neurotransmitter stores (5-HT and NA) in vesicles.

In addition it was recognised that the majority of compounds with therapeutic efficacy in depression have an effect on monoamine neurotransmitter function. Mono-amines are the compounds principally associated with neurotransmission and fall into two main groups - the catechol-amines and the indole-amines. Catechol-amines include noradrenaline and dopamine and the indole-amines include serotonin and melatonin (Aghajanian, 1981).

The tricyclic antidepressant, imipramine, was discovered in 1957 in attempts to treat schizophrenia. While it was ascertained that it did not effectively treat symptoms of schizophrenia, some positive effects were seen in those patients with depression (Everett and Toman, 1959). It was established that imipramine and other tri-cyclic anti-depressants enhance monoamine neuro-transmission function by preventing the reuptake of mono-amines by inhibition of the transporters in the presynaptic neurone at the synaptic cleft (Ho and Estevez, 1982). Further work has established that both electro-convulsive therapy and mood stabilisation e.g. with lithium, alter monoamine function, although how far this can account for their therapeutic actions is not clear.

1.2.3.2 Genetic findings

Twin and family studies have convincingly described a significant familial contribution to aetiology. As a whole, in considering the mood disorders, mono-zygotic twins are seen to have 68% rates of concordance for illness and dizygotic twins have 20% concordance.

When the mood disorders are subdivided, bipolar disorder mono-zygous concordance is 79% and dizygotic concordance is 19%, while the corresponding rates for unipolar disorder are 54% and 20% respectively (Bertelsen et al, 1977; McGuffin et al, 1996).

First degree relatives have an increased likelihood of developing depression, 10-15% in unipolar disorder and 15-20% in bipolar disorder, compared with the normal population prevalence of 1-3% (Gershon et al, 1976). In an attempt to tease out psychosocial and genetic components, adoption studies have been carried out, however these have reported rates of the condition similar to the biological relatives rather than the adoptive families, suggesting the increase is not due to adoption itself or the psychological influences of the adoptive family (Mendlewicz and Rainer, 1974).

With the recognition from the above findings that any model of depression needs to take into account biological factors (Kendler and Karkowski-Shuman, 1997), research has been directed at establishing whether genetic linkage exists according to Mendelian patterns or whether modes of inheritance show gender related differences.

There is therefore no currently accepted autosomal dominant model which is regarded as adequately explaining the genetic findings. As such linkage has been relatively unsuccessful, it is now thought that genetic contributions to aetiology most likely occur because of genes of small effect in a model of polygenic inheritance.

In a similar manner, candidate genes suggested largely through studies of the mono-amine systems have also not been particularly revealing (Cravchik and Goldman, 2000). Findings relating to the serotonin transporter will be summarised later in this chapter.

1.2.3.3 Psychosocial aspects

Based on a line of investigation deriving from the original psychodynamic perspective relating to loss, it has been studied whether major negative life events can precipitate depression. This was established experimentally with a series of studies and it was therefore subsequently recognised that symptomatic “endogenous” depression can also be precipitated by major life events (Brown et al, 1987). The period within which the studies assessed the impact of life events was 6 months prior to relapse although it was also noted that a second negative life event could compound the effects of the first (Paykel, 1969).

Further research has recognised that major life events in a social context may not be independent of the gradual onset of the condition. Subsequent attempts to separate out independent from associated life events (Brown et al, 1987) lead to more recent work which has established that there are not only increased familial rates of depressive illness but that major life events also follow familial patterns. The above findings have therefore led to a complex multi-factorial aetiological framework which includes individual genetic aspects as well as a familial tendency to experience negative life events in a combined gene-environment interactional model (Kendler et al, 1997; Silberg et al, 1999).

1.2.4 Treatment

1.2.4.1 Psychopharmacology

Tricyclic anti-depressants

This group of three ringed molecules plus attached side chains is one of the most highly researched of the antidepressant compounds. After initial successes in the use of imipramine in treating depression a number of other similar compounds have been found to be effective antidepressants. From a clinical view point they are most effectively divided into those with a sedative effect, the tertiary amine group (with terminal methyl groups on the side chain) and those without, the secondary amines. The tertiary amines have a higher affinity for 5-HT re-uptake sites and 5-HT receptors and exhibit greater antagonism of alpha-1 adrenoceptors and muscarinic receptors, the latter effect causing the clinically observed anti-cholinergic side-effects. In spite of their well established efficacy the use of TCA's has been declining recently because of the advent of compounds with improved tolerability and reduced cardiotoxicity (Preskorn, 1993). The latter property means that TCA's must be specifically avoided in patients who are at high risk of impulsive suicide attempts, apart from an exception, lofepramine, which is less toxic. Although reported as having unacceptable side effects by patients, it has been noted that the percentage drop out in controlled treatment studies, when considered by meta-analysis, is only slightly greater than that of SSRIs, and it is known that side effects are the principal cause of drop out from therapeutic studies (Barbui, et al, 2000). However, in clinical situations and over longer periods of treatment the advantage of SSRIs is likely to be greater.

Serotonin specific re-uptake inhibitors

This group of drugs was developed subsequent to the tricyclics in an attempt to produce effective antidepressant compounds that lacked the side effects and over-dose risk of the tri-cyclic anti-depressants. They have a positive efficacy in approximately 60% of patients suffering from depression (reviewed in Edwards and Anderson, 1999) and are relatively safe in overdose, although fatalities have been rarely reported following over-dose with citalopram. They have a specific action at the serotonin transporter preventing reuptake of serotonin from the synaptic cleft following a nerve impulse. Although SSRIs are therapeutically useful, a minority of patients do experience side effects including nausea and GIT effects, dizziness and somnolence. However, these side effects often diminish after the first few days and the antidepressants are therefore generally well tolerated. An increasingly recognised problem is that withdrawal syndromes are possible when they are stopped. This is particularly the case with shorter acting drugs such as paroxetine.

In addition to their use in depression, SSRIs have been widely used in the treatment of OCD, anxiety and panic disorder. There is currently insufficient evidence to delineate clearly the role of serotonin in these disorders as there are numerous neuro-transmitter systems involved and serotonergic neurones appear to have complex effects, some inhibitory, some anxiogenic. It is also known that the introduction of SSRIs clinically can lead to an exacerbation of, or onset of anxiety symptoms before subsequent benefit ensues.

The finding that OCD responds well to SSRIs implies that an underlying abnormality of the serotonergic system might exist, however this cannot be assumed in the absence of a clear demonstration of a connection as, by analogy, Parkinson's Disease (involving abnormal dopaminergic system function), can respond to anti-cholinergic medication.

Studies examining the effect of challenges with agents affecting specific receptors have mainly implicated the 5-HT_{1D} and 5-HT_{2C} receptors (Sasson and Yohar, 1996) (See below).

Mono-amine oxidase inhibitors

This group was actually discovered shortly before the initial use of imipramine as iproniazid is a form of MAOI. They function by inhibiting the mono-amine oxidase enzyme (MAO), which is involved with the intracellular metabolism of noradrenaline, serotonin, tyramine and other amines. In the presence of MAOI's therefore, neuronal stores of these amines are increased, as is their release into the synaptic cleft. These transmitters are then able to positively stimulate post-synaptic receptors for a prolonged period. NA and 5-HT are substrates for Monoamine-A while dopamine and tyramine are metabolised by both monoamine oxidase A and B.

In view of the potential for increased noradrenaline activity to promote a 'pressor' effect, dietary control is needed in patients taking MAOI's, particularly the avoidance of food rich in tyramine as well as sympathomimetic medications. This had led to a reduction in their use except in atypical (Quitkin et al, 1989) or resistant depression (Cowen, 1988).

However, the development of the RIMA, moclobemide, has renewed interest in their clinical utility. They have also been used in the management of severe, unremitting anxiety states (Nutt and Glue, 1989).

Mood stabilisers as anti-depressants

A number of mood stabilisers have been used in the treatment of depression. Their role in the treatment and prophylaxis of bipolar disorder is well established but a number of studies have also concluded that lithium, in particular, is effective in the treatment of depression, both as a primary therapy (Johnson, 1987), as well as in augmentation strategies (Katona and Barnes, 1985) which have been tested in eleven double blind comparative studies with an overall improvement rate of 52% for ten of the studies (reviewed in Fava et al, 2001). Further studies have suggested a similar role for carbamazepine but this is less well supported (Post et al, 1991; Nurnberg and Finkel, 1985). Sodium valproate appears to be more effective in the treatment of manic episodes and their prophylaxis than in the prevention of depressive episodes. The role of Na valproate in the treatment of depression has not been well established. However, for those patients with bipolar disorder who have made a partial response to lithium, the addition of valproate is a further, relatively safe option. Na valproate has also been added to carbamazepine in analogous clinical situations (Post et al, 1991; McElroy et al, 1992). While the mode of action of valproate is not clear, there is some evidence that it can slow the breakdown of GABA, an inhibitory neuro-transmitter. The anti-convulsant lamotrigene has also been used effectively in the treatment of depression in bipolar disorder.

Both carbamazepine and lithium salts appear to facilitate 5-HT function but the precise mechanism for this is not well understood. Other hypotheses regarding the mode of function of lithium include influences on cAMP and second messenger systems or on electrolyte balance, which could have relevance to relapse which may occur in the context of decreased fluid intake or heat exhaustion.

Other anti-depressants

There has been a recent expansion in the number of products available for the treatment of depression with either specific effects or combinations of effects on the mono-amine neuro-transmitter systems. The fundamental requirements of an effective antidepressant, in addition to efficacy, are that it should have few side-effects that develop within the usual dosage range and that it is safe in overdose. Ideally the daily dosage regime should be simple and there should not be a significant withdrawal reaction when the drug is withdrawn.

Venlafaxine, a phenyl-ethylamine derivative, is a new SNRI (Serotonin and Noradrenaline Re-uptake Inhibitor), with some similarities to clomipramine but without sedative or anti-cholinergic side-effects. It has been shown to be effective, particularly in in-patients, in one specific study, where a more rapid onset of action than the comparator, fluoxetine, was reported (Clerc et al, 1994). It is relatively safe in overdose but requires monitoring of blood pressure when the dose exceeds 200 mg per day. Recent studies suggest it is modestly more effective than SSRIs in reducing remission (Entsuah et al, 2001).

Mirtazapine is a NASSA (Noradrenaline and serotonin specific anti-depressant) which blocks 5-HT₂ and 5-HT₃ receptors in a similar way to mianserin, an older anti-depressant that was withdrawn from circulation due to blood dyscrasias. Further pharmacological properties include competitive antagonism at histamine H₁, α -1 and α -2 adrenoceptors. The weaker α -1 antagonism than mianserin is thought to contribute to the ability of mirtazapine to activate 5-HT as well as NA neurones but the H₁ effects unfortunately cause somnolence in 10% of patients taking it. However sleep can be facilitated without daytime drowsiness (Radhakishun et al, 2000). Mirtazapine may potentiate centrally acting sedatives and could theoretically reverse the effects of α -2 adrenoceptor agonists.

Trazodone (a triazolopyridine derivative) is another anti-depressant with 5-HT₂ antagonist properties but the metabolite m-CPP (see below) is a 5-HT agonist. Trazodone also blocks post-synaptic α -1 adrenoceptors and has a sedating profile, but is safer than the TCA's in overdose.

A further new anti-depressant is reboxetine, a morpholine (structurally related to fluoxetine) which is a NARI (Nor-adrenaline re-uptake inhibitor). It is receiving attention as an innovative anti-depressant which is relatively safe and specific. Claims that reboxetine produces an improvement in socialisation have not been fully studied (Dubini et al, 1997). The suggestion that reboxetine boosts patient's energy specifically has also yet to be independently determined and may just be an effect of the resolution of depression itself.

Bupropion is an anti-depressant which is not licensed for this use in the United Kingdom, in view of seizure-related deaths, but it is licensed to assist people withdrawing from nicotine and is widely used in America. The mechanism of action has not been fully elucidated but may partially involve the blockade of DA re-uptake.

1.2.4.2 Electro-convulsive treatment

Five out of six double blind studies that have compared ECT with simulated ECT (sham treatment), such as the MRC trial at Northwick Park (Johnstone et al, 1980) have shown a significant effect of the treatment (Gregory et al, 1985; Brandon et al, 1984; Freeman et al, 1978; West, 1981). One study did not support a beneficial effect of ECT however this was a patient sample where only low dose unilateral ECT was administered (Lambourn and Gill, 1978), a technique now known to be less clinically efficacious.

Other studies have had methodological difficulties including a lack of blinding or small sample size. The use of sham protocols has been necessary to establish that it is not the anaesthetic which is efficacious but that the convulsion is a necessary part of the treatment. Psychopharmacological studies involving neuroendocrine challenge have shown a difference in the post-ECT activity of 5-HT receptors with an up-regulation of 5-HT₂ receptors rather than the down-regulation seen in psychopharmacological studies involving medication, in animals (Gartside et al, 1992).

Down regulation of α -2 and β -1 adrenoceptors is reported in rodent studies post ECT and post anti-depressant treatment, and ECT shows marked effects on dopamine function. Antidepressants can also affect dopamine function but it is difficult to detect unless molecular techniques, such as DA gene expression are used, as has been carried out in animal studies. Of related interest is the work being done using Transcranial Magnetic Stimulation where the convulsion is avoided. These studies have provided some initial support for the use of TMS in depression (George et al, 1999; Cowen, 1999) but the precise indications and long term effects are not well established. It is also not clear whether different cerebral regions need to be targeted for maximum efficacy and whether specific regional stimulation might be appropriate for different psychiatric conditions.

1.2.4.3 Psychological Therapies

In spite of the difficulties researching the efficacy of psychological therapies, psychotherapy has now been widely assessed in randomised control trials. Although there was little empirical evidence for the efficacy of psychodynamic therapy in the past and studies tended to be single case narratives, psychotherapy has now been compared with both drug treatment and waiting lists, with interpersonal psychotherapy (Klerman et al, 1984), being found to improve depressive symptoms in those patients with mild/moderate illness. In one major NIMH study 240 patients were randomly allocated to imipramine, interpersonal therapy, cognitive therapy and placebo.

Statistically significant improvements were found in both the drug treatment and the interpersonal therapy group (Elkin et al, 1989). Although cognitive behaviour therapy did not fare well in this study, it is generally regarded as an effective treatment and importantly may reduce subsequent relapse rates. These findings are in the process of being replicated but a significant body of work has already been summarised in meta-analyses which show an overall support for the effectiveness of psychological therapies in depression, including both psychodynamic and cognitive behavioural treatments (Leichsenring, 2001). As these benefits are not, however always maintained over time (Westen and Morrison, 2001) and are generally more appropriate for less severe illness, it is apparent that current psychological therapies are not sufficiently effective to obviate the need for psychopharmacological intervention. This fact strengthens the rationale for further research into techniques to enhance antidepressant efficacy and speed its onset of action.

1.3 5-HYDROXYTRYPTAMINE

Since its original discovery in 1933 and subsequent description as a 'serum tonic' serotonin (5-HT) has been studied extensively with regard to both the therapeutics and aetiology of psychiatric disorders and numerous lines of investigation now place it centre stage in the study of the neuropharmacology of mood disorders. While depression and its treatment has been an important focus of studies into 5-HT, it has become increasingly clear that the 5-HT system is a complex, widespread neuronal network, malfunction of which may also relate to schizophrenia, anxiety, migraine and the eating disorders.

The complexity of the system relates both to its neuroanatomical distribution and to its numerous receptor sub-types. Originally classified as 'M'(morphine) or 'D'(dibenzyl) receptors (Gaddum and Picarelli, 1957), according to the ability of these substances to directly or indirectly antagonize 5-HT function, molecular biological studies have now characterized seven 5-HT receptor groups, some of which are further subdivided and have been reviewed (Boess and Martin, 1993; Barnes and Sharp, 1999) (See further below).

1.3.1 Structure

Serotonin originally referred to material isolated from the blood stream which was able to constrict smooth muscle and the analogous material purified from the intestine was known as 'enteramine'. Crystallization and later analysis showed both substances to be the same and synthetic manufacture followed. The molecule is an indole and structurally related to LSD and other psychotropic molecules (Cooper et al, 1986).

1.3.2 Neuro-anatomical distribution

The majority of 5-HT neurones arise from the raphe nuclei - the dorsal raphe nucleus (DRN), median raphe nucleus (MRN) and B9 which is in the rostral brain stem (See Figure 1.1). 5-HT neurones have widespread connections to other parts of the central nervous system, ascending to the fore-brain via the median fore-brain bundle, located in the lateral hypothalamus. From there the neurones extend out to the limbic system including the amygdala, hippocampus and cingulum as well as to the neocortex and ventrolateral cortical regions.

Serotonergic projections from the MRN are found mainly in the hippocampus and septum. These axons differ morphologically from those arising in the DRN which project to the cortex and striatum.

It has been found in animal studies that 5-HT projections from the dorsal raphe are most closely associated with 5-HT₂ receptors (Kosofsky and Molliver, 1987).

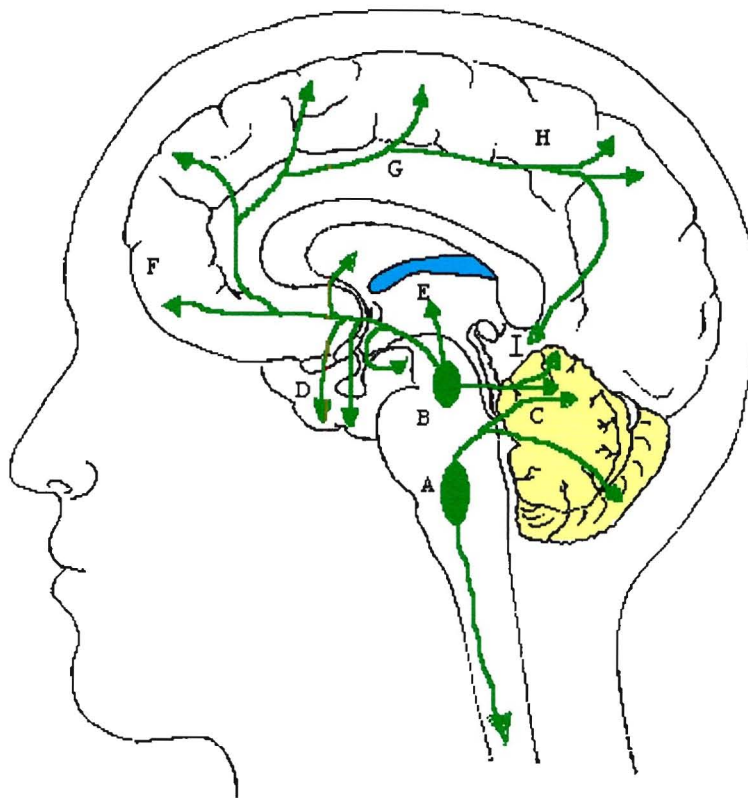


Figure 1.1: Diagrammatic representation of the major serotonergic tracts in the human brain. A= Median raphe nuclei; B= Dorsal raphe nuclei; C= Deep cerebellar nuclei; D=Limbic structures; E= Thalamus; F=Neocortex; G=Cingulum; H= Cingulate gyrus; I=To hippocampus. Cells arising from the raphe nuclei project to all of the cortical grey matter. Further tracts extend to the basal ganglia and cerebellum.

From the above it can be seen that projections from the major collections of serotonergic cell bodies are not distributed homogeneously but that differing neuronal types and regions of distribution may lead to varying ratio's of receptor sub-types and contribute to functional specialisation , by region and morphology.

It has therefore been noted that this pattern of distribution, according to particular subtypes, may underlie development of a modular system with a degree of neuro-pharmacologically specific activity and it is therefore possible that refining pharmacological manipulation will be able to influence behavioural outcomes (Cowen, 1993).

1.3.3 Neuro-transmission

1.3.3.1 Synthesis and metabolism

Serotonin is synthesised in the brain from the amino acid tryptophan (TRP) via the 5-hydroxyindole pathway as it cannot cross the blood-brain barrier (Lovenberg et al, 1968). TRP is an essential amino acid and is therefore obtained in the diet or, failing that, from the catabolism of body protein (Wurtman, 1978). TRP enters the brain through the blood brain barrier in common with other amino acids such as tyrosine and phenylalanine (aromatic amino acids) and isoleucine, leucine and valine (branch chain amino acids) by a common transportation carrier.

However, TRP competes with these other large neutral amino acids for transport into the brain and it is therefore the ratio of TRP to the other amino acids which regulates the quantity of tryptophan entering the brain (Wurtman, 1980). In the blood, approximately 90% of tryptophan is usually protein-bound to serum albumin while the remainder is free tryptophan (unbound).

As can be seen in Figure 1.2 synthesis of serotonin (5-HT) is controlled by hydroxylase enzymes. The first step in the formation of 5-HT from the amino acid tryptophan is hydroxylation to 5-Hydroxytryptophan (5-HTP). The enzyme involved in this transformation is tryptophan hydroxylase which is located within serotonergic neurones (Knott and Curzon, 1972). This enzyme acts as the rate limiting step in serotonin synthesis. Synthesis is finally completed by the conversion of 5-HTP to 5-HT, by a decarboxylase enzyme.

The enzyme activity of this decarboxylase enzyme is greater than the activity of the hydroxylase involved in the rate limiting step and it is therefore understandable that little free 5- HTP is found in the mammalian brain (Fernstrom and Fernstrom, 1995).

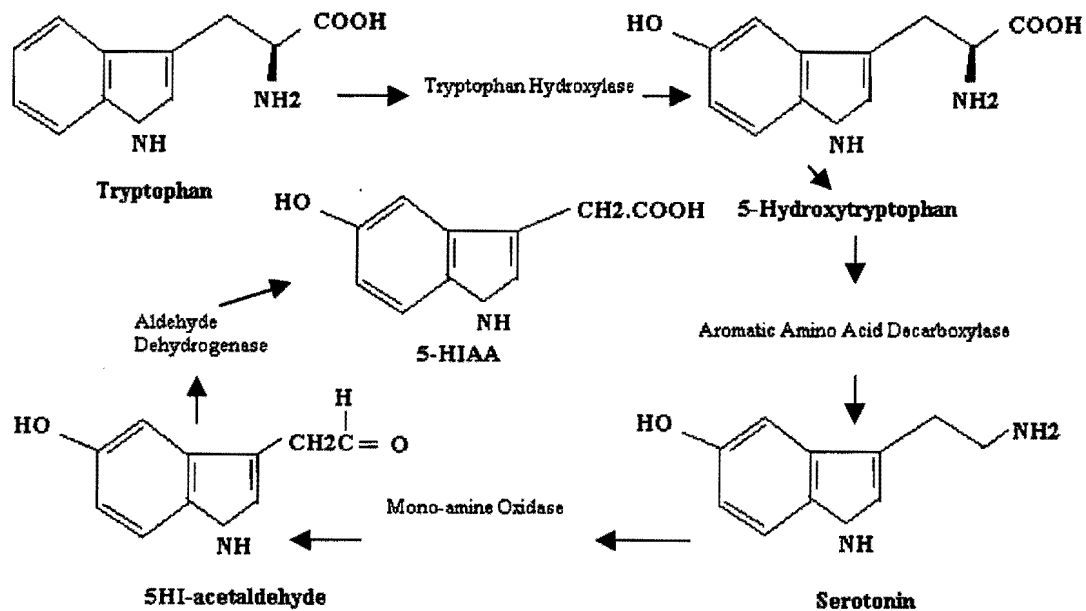


Figure 1.2: Diagrammatic representation of serotonin production and metabolism. Some alternative metabolic pathways have not been demonstrated.

Following synthesis, 5-HT is stored in granules at the pre-synaptic nerve terminals, awaiting the stimulus for release. There is a major cascade on arrival of a nerve impulse with thousands of neuro-transmitter molecules being present in every neurotransmitter terminal vesicle and there are also multiple vesicles in the pre-synaptic terminal. As can be seen in Figure 1.2, metabolism of 5-HT occurs by initial oxidation to 5-Hydroxyindole-acetaldehyde by monoamine oxidase (MAO) followed by dehydrogenation to 5-HIAA by aldehyde dehydrogenase. An alternative pathway involves the conversion of 5-Hydroxyindole-acetaldehyde to 5-Hydroxytryptophol by aldehyde reductase (Cheifetz and Warsh, 1980). Other minor metabolic pathways exist including a conjugation pathway to a sulphate derivative and action by methyltransferase with the production of methylate compounds.

1.3.3.2 The 5-HT transporter

The serotonin transporter is a molecule which has an important function in the regulation of serotonergic transmission as it controls the uptake of serotonin into the presynaptic neurone and is therefore an early site for the action of both anti-depressant drugs and those with a harmful or neuro-toxic action (Amara and Kuhar, 1993). Both tri-cyclic anti-depressants and SSRIs are considered to function by the occupancy of binding sites adjacent to and overlapping with the substrate binding site. In addition to this positive effect in drug treatment, certain neuro-toxins, such as substituted amphetamines are concentrated in 5-HT neurones as a result of the transporter action (Lesch et al, 1995).

The serotonin transporter is also found in the human blood platelet membranes where it has been used as a model in numerous investigations of 5-HT re-uptake. These investigations have shown a reduced rate of serotonin transporter in the platelets of depressed patients (Coppen et al, 1978; Stahl et al, 1983) and also reduced binding of (³H)imipramine and (³H)paroxetine (Paul et al, 1981; Briley et al, 1980).

1.3.4 Receptor subtypes

After the original division of the serotonin receptors by Gaddum and Picarelli (1957), into M and D receptors, subsequent classifications were based on demonstrable functional differences by the use of radioligands and other pharmacological probes.

This was initially useful and the 5-HT₁, 2 and 3 receptors were described, with additional subtypes of the 'type 1' receptor being characterised by radioligands and second messenger systems. This allowed inclusion of 5-HT_{1C} with the 5-HT₂ group as 5-HT_{2C}. After the discovery of the 5-HT₄ receptor, subsequent divisions resulted from the application of molecular techniques to the task.

Table 1.2: Classification of serotonin receptor subtypes, including information regarding chromosomal origin and effects of agents on hormonal responses.

Serotonin receptor	Amino acids	Human chromosome	Homology (%) with 5-HT _{1A}	Agonist	Antagonist	Hormonal /neuro-transmitter responses
5-HT _{1A}	422	5q11.2-Q ₁₃	100	Buspirone 8-OH-D.PAT Gepirone	WAY-100635	ACTH, Cortisol, PRL, GH release
5-HT _{1B}	390	6q 13	43	RU 24969	Pindolol	-
5-HT _{1D}	377	1q 34.3-36.3	43	Sumatriptan PNU 109291	Ketanserin Ritanserin	Ac. Ch release.
5-HT _{1E}	365	6q14-q15	40	-	-	-
5-HT _{1F}		3q11	42	LY334370 CY344864		-
5-HT _{2A}	471	13q 14-21	30	DOI	MDL 100907 Ketanserin	Cortisol, ACTH, rennin, prolactin release
5-HT _{2B}	481	2q 36.3-37.1	34	5-HT (full) TFMPP quipazine (partial)	SB 200646 SB 204741	-
5-HT _{2C}	458	X	32	m-CPP	Mesulergine SB242084	PRL release
5-HT ₃	487	11	14	SR 57227	ondansetron	Inhibits cortical Ac.Ch release
5-HT ₄	387	5q 31-33	29	BIMU 8 RS 67506	SB 204070 RS 100235	5-HT, CCK and Ac.Ch release
5-HT _{5A}	357	7q 36	36	-	-	5-HT, DA and Ac.Ch. release
5-HT _{5B}	± 370	2q 11-13	39	-	-	-
5-HT ₆	440	1p 35-36	34	-	Clozapine	-
5-HT ₇	445-448	10q 21-24	38	-	SB 269970	-

There are therefore now 7 families of serotonin receptors, some of which have been further divided into additional sub-types based on structural, operational and transduction information according to the principles of the Nomenclature Committee of the International Union of Pharmacology (Hoyer et al, 2002). The chromosomal origin and information regarding relevant agonists, antagonists and responses are summarised in Table 1.2.

1.3.4.1 The 5-HT₁ family.

The previous nomenclature included three subtypes within this group 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C}. Subsequent work has delineated the similarity of the 5-HT_{1C} receptor to the type 2 family and the 5-HT_{1C} receptor was therefore re-named the 5-HT_{2C} receptor. Initial typing of the 5-HT₁ family was based on the affinity of ^{3H}-5-HT for rat cortex binding sites (Peroutka et al, 1979). Further 5-HT₁ receptors which have been recognised include the 1D, 1E and 1F receptors. The receptors of the 5-HT₁ family all couple negatively to adenylate cyclase via G-proteins and have high amino acid sequence homology.

(Reviewed in Barnes and Sharp, 1999). 5-HT₁ receptors act through the stimulation of cAMP synthesis.

5-HT_{1A} receptors

This receptor is the most well characterised of all of the 5-HT receptors. There are several reasons for this including the discovery of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (Hjorth et al, 1982).

Another reason has been potential clinical applicability, as 5-HT_{1A} agonists such as buspirone have been found to be anxiolytic and have been used in augmentation strategies in the treatment of depression (Blier and Bergeron, 1998).

The rat 5-HT_{1A} receptor has 422 amino acids and has 89% sequence homology with the human receptor which is located on chromosome 5 (5Q 11.2 - Q13). Both autoradiographic and PET studies have provided us with information regarding the distribution of receptors in the brain. The density is high in limbic areas especially hippocampus, lateral septum and cortical areas including the cingulum and entorhinal cortices as well as the raphe nuclei where the receptor acts as an inhibitory autoreceptor.

There are areas with a lower frequency of binding sites in other parts of the brain including the cerebellum and basal ganglia. The 5-HT_{1A} receptors are located post-synaptically in fore-brain regions and on 5-HT neurones in the raphe nuclei.

Animal studies have shown that administration of 5-HT_{1A} receptor agonists causes a range of behavioural and physiological effects including hyperphagia, hypothermia, altered sexual behaviour and a tail flick response (Green and Grahame Smith, 1976). One study involving chronic administration of m-CPP, which has a greater effect on the 5-HT_{2C} receptor (See further below), showed no effect on either the pre- or post-synaptic 5-HT_{1A} receptors (Aulakh et al, 1991).

Neuroendocrine studies have found that 5-HT_{1A} receptor agonists cause ACTH and corticosteroid increase (Gartside et al, 1990), as well as electrophysiological and neurochemical changes (Clifford et al, 1998). In humans increases in growth hormone are also seen (Cowen, 1990). These endocrine responses may be blocked by 5-HT_{1A} receptor antagonists (Cowen, 1990; Gartside et al, 1990).

5-HT_{1B} receptors

It was established that this receptor was distinguishable from 5-HT_{1A} and 5-HT₂ receptors due to the low affinity for 8-OH-DPAT (Middlemiss and Fozard, 1983). In the rat brain this receptor was characterised as having low affinity for spiperone (Pedigo et al, 1981) and a species variant, termed 5-HT_{1D} initially, was found in bovine brain (Heuring and Peroutka, 1987), as well as in humans. Cloning studies have established that one of the 2 human variants of the 5-HT_{1D} receptor (5-HT_{1Dβ}) is a species equivalent of the rat 5-HT_{1B} receptor with 96% sequence homology (Jin et al, 1992). Since the pharmacology of the species variants can differ, prefixes are used to denote species specific receptors e.g. human = h5-HT_{1B}. The genes encoding these receptors are located on chromosome 9, mouse, and on chromosome 6 (6q, 13), human, respectively. (Saudou and Hen, 1994).

As regards distribution in the rat brain, a high density of 5-HT_{1B} sites is found in the basal ganglia as well as in other regions (Pazos et al, 1985). m-RNA has been located in the dorsal and median raphe nuclei in the rat as well as in some forebrain sites.

It is likely that the 5-HT_{1B} receptors are located both pre- and post- synaptically, as well as possibly on some non-5-HT neurons. They are also likely to function as both auto- and hetero-receptors as discussed below. In situ hybridisation has established that m-RNA is localised to hippocampal pyramidal and granule cells and, in the caudate, to medium spiny neurones.

Originally, ligands available for use as probes of 5HT_{1B} function were not selective but more recently specificity has improved and high affinity has been reported for the antagonists SB-224289 and SB-216641 (Roberts et al, 1997). As mentioned previously, there are species differences in the pharmacology of equivalent receptors e.g. pindolol, a β -adrenoceptor antagonist, has a higher affinity for the rodent 5-HT_{1B} receptor than the human receptor. In a similar manner to a number of other 5-HT receptors, it has been established that second messenger responses occur by the negative coupling of 5-HT_{1B} receptors to adenylate cyclase under forskolin - stimulated conditions (Adham et al, 1992). From a functional perspective it has become clear that the 5-HT_{1B} receptor has auto-receptor activity at serotonergic nerve terminals (Buhlen et al, 1996). Some data also suggest the presence of 5-HT_{1B} auto-receptors in the DRN.

According to m-RNA presence, 5-HT_{1B} receptors may be found in the DRN but possibly also on 5-HT neurones terminating at the DRN, (Starkey and Skingle, 1994). In addition to an autoreceptor role 5-HT_{1B} receptors are also thought to have a heteroreceptor role and regulate the release of other neurotransmitters.

This has been suggested for inhibition of acetyl-choline release (Maura and Raiteri, 1986) and facilitation of dopamine release by 5-HT_{1B} agonists (Lyer and Bradberry, 1996) in the frontal cortex. 5-HT_{1B}-heteroceptors are also described as underlying the suppression of GABA_B - mediated IPSP's in rat midbrain dopamine neurones, in vitro (Johnson et al, 1992).

From a behavioural perspective, an involvement of 5-HT_{1B} receptors in mouse locomotor responses is now better supported by stimulant studies and the absence of this response in knock-out mice (Saudou et al, 1994), than previous work suggesting a role in the rat locomotor response (Green and Heal, 1985). More precise functional effects on hormonal and physiological measures still need to be clarified but studies have reported a negative effect of 5-HT_{1B} agonists, such as TFMPP and RU24969, on appetite, with an induced hypophagic effect (Kennett et al, 1987; Kitchener and Dourish, 1994). Although knock-out mice are more aggressive than wild type mice, 5-HT_{1B} antagonists are not generally regarded as substances promoting aggression. In a further study involving knock-out mice, a 5-HT_{1B} agonist – CP 93129, inhibited labelled 5-HT release from control hippocampal and cortical slices but not from mutants (Piñeyro et al, 1995).

A further behavioural response which has been observed is contralateral rotation in the guinea pig following agonist injection into the substantia nigra, where high levels of 5-HT_{1B} receptors are found on the terminals of striatonigral GABA neurones.

Other possible responses include agonist induced hypothermia and the potentiation of 5-HTP induced myoclonic jerks (Hagan et al, 1995).

5-HT_{1D} receptors

As discussed earlier, the previously named 5-HT_{1D} receptor is actually a species equivalent of the rodent 5-HT_{1B} receptor and was initially characterised from work on bovine and human brain tissue (Heuring and Peroutka, 1987). Subsequently, molecular techniques have revealed a further receptor with 5-HT₁ homology, and this is now the 5-HT_{1D} receptor proper (Hartig et al, 1996).

The human 5-HT_{1D} receptor gene is located on chromosome 1p34.3 - 36.3. In the rat brain 5-HT_{1D} receptors are found in the basal ganglia, hippocampus and the cortex (Bruinvels et al, 1993) while in the human brain, in addition to the basal ganglia, receptors are found in the mid-brain and spinal cord (Castro et al, 1997).

Although found in similar regions with in situ hybridisation studies, m-RNA was not detected in globus pallidus, ventral pallidum and substantia nigra, suggesting, with other evidence, that the 5-HT_{1D} receptor is largely based presynaptically - on axon terminals.

The pharmacology of the 5-HT_{1B} and 5-HT_{1D} receptors is similar in humans and in other species, apart from the rat 5-HT_{1B} receptor. In humans, ketanserin and ritanserin have some selectivity for the 5-HT_{1D} receptor, (Pauwels et al, 1996) and exhibit antagonist properties.

It is not yet clear which second messenger response can be linked with the 5-HT_{1D} receptor in native tissue but in transfected cells cloned receptors couple negatively to adenylate cyclase, as is seen with other 5-HT receptors. In a similar manner to the 5-HT_{1B} receptor an additional auto receptor role has been proposed for the 5-HT_{1D} receptor, however agonist-induced 5-HT inhibition in 5-HT_{1D} knock-out mice has been both confirmed and refuted (Piñeyro et al, 1995). Further research with more specific drugs is needed to confirm both an auto-receptor role and various heteroreceptor possibilities. The latter include the inhibition of glutamate release in both rats and humans (Maura et al, 1998) and an inhibitory effect on GABA in humans (Feuerstein et al, 1996). Other findings have included influences on acetyl-choline release. Low levels of 5-HT_{1D} compared to 5-HT_{1B} receptors and poor brain penetration have complicated attempts to assign functional responses to the 5-HT_{1D} receptor but it is of interest that one recent paper has described a reduction in sensitivity of these receptors in patients with melancholic depression (Whale et al, 2001). Further work involves the testing of agonists with putative anti-migraine effects, such as PNU-142633 (McCall RB et al, 2002).

5-HT_{1E} receptors

The finding of a biphasic response curve when 5-CT application followed the introduction of tritiated 5-HT, suggested that more receptors than the 5-HT_{1D} receptor (high affinity) existed. It was the 5-HT_{1E} receptor which was shown to have a low affinity in these ligand binding studies. Although other receptors could have demonstrated similar findings the 5-HT_{1E} gene was then subsequently isolated by molecular techniques.

The gene encodes a 365 amino acid protein and is intronless. It is located on human chromosome 6q 14-q15 (Levy et al, 1992).

It can be inferred from data from a number of sources that the 5-HT_{1E} receptor is located post-synaptically. This is suggested by auto-radiographic studies showing that lesions of 5-HT neurons lead to no variation in 5-HT_{1E} binding sites in the rat forebrain. (Barone et al, 1993). Additional support for the above is that 5-HT_{1E} m-RNA is present in the monkey and human brain in cortical areas, the putamen and the caudate. Somewhat lower levels are detected in the amygdala and hypothalamic regions, a pattern similar to that seen with 5-HT_{1B} and 5-HT_{1D} m-RNA (Bruinvels et al, 1994a and b). There is as yet, however, no evidence that the raphe nuclei contain 5-HT_{1E} m-RNA. The absence of a 5-HT_{1E} selective ligand has complicated attempts to work out its distribution and function. In addition to the m-RNA studies described above, distribution is inferred from areas of non-5-HT_{1A/1B/1D/2C} [³H] - 5-HT binding in human and other species. These include cortical areas, especially entorhinal but also caudate, putamen and claustrum, hippocampus and amygdala. (Miller and Teitler, 1992).

From a pharmacological perspective the 5-HT_{1E} receptor is characterised by low affinity for 5-CT and higher affinity for 5-HT. In this respect it is similar to the 5-HT_{1F} receptor but they are differentiated by the lower affinity of the 5-HT_{1E} receptor for sumatriptan.

The physiological role of the 5-HT_{1E} receptor has not been clarified however one functional capacity which has been established is the mediation of a moderate inhibition of forskolin stimulated adenylate cyclase (Adham et al, 1994b).

In studies of radioligand binding 5-CT has a very low potency as an agonist while methiopin behaves as a weak agonist (Adham et al, 1994a).

5-HT_{1F} receptors

The 5-HT_{1F} receptor was initially termed the 5-HT_{1EB} receptor as it showed low affinity for 5-CT (Similar to the 5-HT_{1E} receptor) but differed in distribution from the 5-HT_{1E} mRNA, (Amlaiky et al, 1992). Following characterisation of the mouse 5-HT_{1F} receptor as having high sequence homology with other type I receptors - the 5-HT_{1B} and 5-HT_{1D} receptor, the human receptor was itself sequenced (Adham et al, 1993b) and located on chromosome 3q11. Following in-situ hybridisation studies, m-RNA was located at significant rates in the mouse and guinea-pig brains in the hippocampus (CA1 - CA3 layers), cortex and DRN. Human studies have been carried out where correlation was seen with m-RNA distribution in the guinea-pig i.e. binding at the highest levels was found in cortical and hippocampal areas, claustrum and the caudate nucleus. 5-HT_{1F} receptor binding sites are low in the substantia nigra (Waeber et al, 1995).

Unlike the 5-HT_{1E} receptor, the 5-HT_{1F} receptor has a high affinity for sumatriptan. However, they both have high affinity for 5-HT and low affinity for 5-CT (Amlaiky et al, 1992). As with other 5-HT₁ receptors, the human and mouse receptors couple to the inhibition of forskolin - stimulated adenylate cyclase. 2 novel agonists have been reported - LY334370 and LY344864, (Johnson et al, 1997). Studies with these two novel agonists have not, however, supported clear behavioural profiles in rats.

The function of the 5-HT_{1F} receptor is not known but it may be involved in both visual and cognitive roles and as an auto-receptor (Waeber et al, 1995).

1.3.4.2 The 5-HT₂ family

Activation of both 5-HT_{2A} and 5-HT_{2C} receptors stimulates phosphoinositide hydrolysis. This fact, and the low divergence of amino acid sequence homology between the type 2 receptors (60% homology between the three subtypes) lead to their current classification grouping. This family therefore includes the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors.

They display numerous similar features including structure - a high level of amino-acid sequence homology is seen in the 7 transmembrane domains (Baxter et al, 1995); function - all couple positively to phospholipase C and stimulation leads to the mobilisation of intra-cellular calcium; genetic structure - all have either 2 or 3 introns in the coding sequence (Chen et al, 1992); and pharmacology- a relatively low affinity for 5-HT, a high affinity for the 5-HT₂ receptor agonist DOI and a high affinity for other 5-HT₂ receptor antagonists including Ritanserin. The 5-HT_{2A} receptor was previously known as the 5-HT₂ receptor and the 5-HT_{2C} receptor was previously the 5-HT_{1C} receptor.

The 5-HT_{2B} receptor was termed the 5-HT_{2F} or SRL receptor in previous studies and had been originally related to the 5-HT₂ receptor as a 5-HT₂-like receptor in the fundus of the rat stomach.

5-HT₂ receptors have all been shown to desensitise after being exposed to 5-HT and other agonists for prolonged periods although the precise modes of this loss of function may differ (Sanders-Bush, 1990).

5-HT_{2A} receptors

Originally termed the 5-HT₂ receptor, the 5-HT_{2A} receptor was subsequently grouped with the 5-HT_{2C} and 5-HT_{2B} receptors in the 5-HT₂ family on the basis of having a similar pharmacological profile and second messenger system. It was later confirmed that the three receptors were also closely structurally related with high amino-acid sequence homology, although greater between 5-HT_{2A} and 5-HT_{2C} than between 5-HT_{2A} and 2B.

5-HT_{2A} receptors were initially characterised as 5-HT receptors with a high affinity for [³H]-spiperone and low affinity for serotonin itself. (Leysen et al, 1978).

The human 5-HT_{2A} receptor is located on chromosome 13 q14- q21. Specific amino-acids have been studied within the receptor which are available for glycosylation and phosphorylation and others have been shown to affect ligand binding and effector coupling (reviewed in Boess and Martin, 1994).

A number of different study methods have ascertained the distribution of the 5-HT_{2A} receptor in the human brain, including auto-radiography with [³H]-spiperone and [¹²⁵I]-ketanserin, in-situ hybridisation and immunocytochemistry. In addition it is well established that 5-HT_{2A} receptors are present in the same location as the cells expressing them by m-RNA distribution (Mengod et al, 1990; Burnet et al, 1994).

High levels are found in numerous forebrain and cortical regions as well as in the caudate nucleus, nucleus accumbens and some limbic areas. (Pazos et al, 1985).

This distribution also corresponds to that of axons arising from the DRN (Blue et al, 1988), and the predominant neuronal location of the receptor is supported by other mRNA studies (Burnet et al, 1995). Evidence has also shown receptor activity on GABA-ergic interneurons (Morilak et al, 1993) and glutamatergic pyramidal neurons (Burnet et al, 1995).

Original attempts to delineate functional attributes of the individual 5-HT₂ receptors were hampered by the lack of selective agents, a situation which is now improving with the development of highly selective antagonists such as MDL 100907 (Sorensen et al, 1993) and SB 200646A (and others) which distinguish between the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (Baxter, 1996). Work on agonists is relatively less advanced.

As regards second-messenger responses, 5-HT_{2A} receptors have been shown to activate phospholipase-C through G-protein coupling (Sanders-Bush et al, 1995), as mentioned above. In different studies agonists such as DOI have exhibited partial agonist properties, m-CPP displays 5-HT_{2A} antagonist activity and other ligands act as inverse agonists.

A further interesting finding is the evidence that altered gene expression can follow 5-HT_{2A} receptor stimulation, as exemplified by brain-derived neurotrophic factor (BDNF) (Vaidya et al, 1997).

Other evidence for a downstream regulatory rôle of other systems comes from electrophysiological studies suggesting a 5-HT_{2A} role in the regulation of NA neurones.

There is also evidence from micro-dialysis studies (Done and Sharp, 1994) that 5-HT₂ antagonists increase NA release. Whether this is a 5-HT_{2A} or 5-HT_{2C} receptor-mediated event activity is not currently clear. Behavioural responses to 5-HT_{2A} receptor activation are now more reliably known to include rat 'wet dog shakes' and mouse 'head twitches' (Green and Heal, 1985). A close link between hallucinogenic potency and 5-HT₂ binding has led to a search to discriminate whether 5-HT_{2A} or 5-HT_{2C} receptors are more involved. Although both correlate, the predominantly 5-HT_{2C} agonist m-CPP is not hallucinogenic.

The 5-HT_{2A} binding site has a high affinity for anti-psychotic drugs and interest in the link with psychosis has been increased following the correlation of 5-HT_{2A} polymorphic variants with treatment response (Busatto and Kerwin, 1997). Other functional responses include hyperthermia, and increased secretion of cortisol, ACTH, renin and prolactin (Bagdy, 1996).

5-HT_{2B} receptors

In 1959, Vane established that serotonin induced contraction of the rat stomach fundus. Subsequent work linking this action to a specific receptor suggested that the effect was mediated by a 5-HT₁-like receptor (Baez et al, 1990).

Although the receptor behaved in a similar pharmacological way to the subsequently named 5-HT_{2C} receptor, 5-HT_{2C} receptor m-RNA was not detected in the same location. Molecular genetic studies subsequently established the identity of a similar receptor, initially termed the 5-HT_{2F} receptor but renamed later as the 5-HT_{2B} receptor (Humphrey et al, 1993), and located the gene on chromosome 2 from q36.3-37.1. The receptor is 481 amino acids in length and shows significant sequence homology with both the 5-HT_{2A} and 5HT_{2C} receptors. As discussed above, the 5-HT_{2B} receptor is similar to the other members of the 5-HT₂ family from a genetic perspective: the gene has two introns which correspond to those in the 5-HT_{2A} and 5-HT_{2C} genes (Foguet et al, 1992b). The distribution of the receptor in the brain is more limited than other members of the family, being found in the cerebellum, lateral septum, dorsal hypothalamus and medial amygdala. The receptor binding of the 5-HT_{2B} receptor is similar to the 5-HT_{2C} and 5-HT_{2A} receptors, although it has lower affinity for ritanserin and higher affinity for yohimbine (Kennett et al, 1994b).

Some species differences in pharmacology seem to exist but less so between the rat and human (Bonhaus et al, 1995). From a functional perspective, the cloned rat and human 5-HT_{2B} receptors stimulate phosphatidylinositol hydrolysis. In functional models 5-HT itself and analogues behave as full agonists while TFMPP and quipazine are partial agonists.

5-HT_{2B} receptors may regulate mitogenic effects during neural development (Choi et al, 1997). Apart from a possible anxiolytic effect there is limited information on behavioural effects. (Duxon et al, 1997).

5-HT_{2C} receptors

The 5-HT_{2C} receptors are distributed extensively throughout the brain but the greatest density is found in the choroid plexus (Pazos et al, 1987). Lower densities are found in the hippocampus, amygdala, cingulate, corpus striatum, subthalamic nucleus, supra-chiasmatic nucleus and the grey matter of the spinal cord. The relevance of this receptor to psychiatric illness and its treatment will be discussed further in subsection 1.5 and further aspects of the receptor itself will be detailed in subsection 1.7.2.1.

1.3.4.3 The 5-HT₃ Receptor

Unlike the other 5-HT receptors, the 5-HT₃ receptor is a ligand-gated ion channel. From a molecular perspective, only one gene, encoding the 5-HT_{3A} subunit, has been characterised (Maricq et al, 1991). This sub-unit is formed from 487 amino-acids and displays greater similarity to members of other receptor super-families such as the nicotinic and glycine receptors. Two forms of the sub-unit have been characterised- the short form, 5-HT_{3S} being reported in a number of species (Reviewed in Barnes and Sharp, 1999) but the m-RNA of the long form is not expressed in humans (Werner et al, 1994). The short form results from a deletion of 18 nucleotides in exon 9 of the gene and in humans the gene is found on chromosome 11 (Uetz et al, 1994). The ion channel is formed by a lining of 5 sub-units, with gating by an inward bend at a hydrophobic leucine residue, in common with other similar receptors (Unwin et al, 1993). The N- and C-terminals of the subunit are extracellular and the channel itself is 3 nm in diameter (Boess et al, 1995) and selective for cations.

The 5-HT₃ receptor is mainly found in the brainstem. By comparison, forebrain expression is generally low, but there is both species and regional variation, with higher levels in some limbic areas and in humans – high levels are seen in the caudate and putamen (Abi-Dargham et al, 1993). In rodents, relatively higher cortical expression is seen than in humans. A relatively high level is seen in the hippocampus in most species (Parker et al, 1996), and m-RNA is mainly found in the inter-neurons. Data from different sources supports the role of the receptor in the activation of GABA inter-neurons, leading to downstream inhibition of pyramidal neurones (Tecott et al, 1993). These GABA neurones, in the human basal ganglia, are also those that are seen to degenerate in Huntington's disease (Steward, 1993).

The pharmacological aspects of the receptor have been noted for their inter-species variation as well as for the receptor's capacity for allosteric modulation by numerous compounds which also affect other superfamily members. Clinically relevant compounds include alcohols, steroids and anaesthetic barbiturates (Miller et al, 1996).

There has been some evidence for the further delineation of 5-HT₃ receptor subunits since the cloning of 5-HT_{3A} by current/voltage relationships rather than pharmacological means in one case (Hussy et al, 1994) and further conductance studies by another group (Yang et al, 1992). These additional subunits were suspected by the functional diversity of the receptor and may interact with other receptor subunits.

From a functional perspective, 5-HT₃ receptors in the dorsal vagal nuclei in the brainstem are considered important in the anti-emetic action of the 5-HT₃ antagonists. Initial hopes of a use for antagonists in a number of different kinds of psychiatric disorder, have not been supported well, although animal models have often suggested an anxiolytic action of these compounds.

The relationship of 5-HT₃ to CCK and the capacity of CCK to induce panic further supports this link. Unfortunately, other than a possible elevation of pain threshold, knockout mice have not thrown light on this and other questions (Guy et al, 1997).

Some early work has supported a positive effect of antagonists such as ondansetron on cognitive performance and this has also been seen with other antagonists, although not uniformly between groups (Bentley and Barnes, 1995). One possible mechanism for this effect is that 5-HT₃ stimulation decreases cortical acetyl choline release, thereby supporting the reverse i.e: enhancement of cognition by 5-HT₃ antagonism (Consolo et al, 1994). This is further supported by the fact that acetyl choline activity has been correlated with cognitive performance and Ac-Ch deficits, such as are seen in Alzheimer's Disease, reduce performance (Bartus et al, 1982).

A further role of the receptor relates to its capacity to influence dopamine activity in central brain regions, by facilitatory mechanisms. This is shown in rat studies following receptor activation in the nucleus accumbens and striatum (Blandina et al, 1988).

Other studies have used electrochemical techniques to investigate effects on the dopamine system; however it would seem, overall, that 5-HT₃ antagonists do not alter basal metabolism or release of dopamine (Koulu et al, 1989).

1.3.4.4 The 5-HT₄ Receptor

It has been known for some time that serotonin is able to stimulate adenylate cyclase in the brain (Fillion et al, 1975). This and other functional responses due to the activity of the 5-HT₄ receptor have been demonstrated in animal studies, centrally in mouse colliculi neurones and guinea pig brain and also in peripheral tissues (reviewed in Ford and Clarke, 1993). Two species of the receptor have been sequenced, short or 5-HT_{4(a)} and long or 5-HT_{4(b)}, which arise by alternative splicing of the m-RNA from position 360 (Hoyer and Martin, 1997). There are two further splice variants and these have been confirmed in animal and human genomes - 5-HT_{4(C)} and 5-HT_{4(D)} with common sequences up to amino acid 358. The receptor gene has been located on chromosome 5 between 5q31 and 5q33 (Cichon et al, 1998). The 5-HT_{4A, B} and C isoforms are found in both the brain and the GIT while 5-HT_{4D} receptors have only been located in gut tissue (Blondel et al, 1998). Both receptors and m-RNA in the brain are found chiefly in nigrostriatal and mesolimbic areas. All 4 receptors couple positively to adenylate cyclase but differences in efficiency are likely following phosphorylation and this process is probably also related to desensitisation of the 5-HT₄ receptor (Ansanay et al, 1992). One of the functional attributes of the 5-HT₄ receptor is through the modulation of other neurotransmitters including acetyl - choline.

This capacity has been supported with both agonist and selective antagonist studies, (Consolo et al, 1994). Post mortem brain samples have also shown reduced hippocampal 5-HT₄ receptor density in Alzheimer's patients (Reynolds et al, 1995). In addition to the modulatory effect on the cholinergic system, evidence exists for a role in dopamine modulation, with both in vitro and in vivo studies showing increased striatal dopamine release, by indirect means, as 5-HT₄ receptors seem to be found, not on dopamine neurone terminals, but on GABA-ergic terminals of neurones with striatal cell bodies (Gerfen, 1992).

In addition to the modulation of other neuro-transmitters, 5-HT release itself is modulated by the 5-HT₄ receptor. Microdialysis techniques have revealed that activation of 5-HT₄ receptors leads to serotonin release from the hippocampus. This release was further modified by 5-HT₄ antagonists by inhibition, an effect also seen in the substantia nigra (Thorre et al, 1998).

In spite of laboratory evidence for the above modulatory effects, no behavioural effects are seen either with administration of drugs affecting 5-HT₄ function or behaviours related to variations in Dopamine function (Reavill et al, 1998). This finding is also supported in primate studies (Terry et al, 1998). While the modulatory effect of 5-HT₄ activity on Acetyl Choline may provide the link with enhanced memory, it is also known that 5-HT₄ receptors are found in hippocampal pyramidal neurones and thus might implicate long term potentiation as a mechanism (Ullmer et al, 1996).

Evidence is currently lacking in humans for a cognitive enhancing effect of 5-HT₄ stimulation. From a psychiatric perspective, 5-HT₄ may play a role in anxiety however the findings have been contradictory, with animal models showing both evidence for anxiolysis (Kennett et al, 1997) and inhibition of benzodiazepine anxiolysis (Costall and Naylor, 1992) by 5-HT₄ antagonists. Interesting parallels are evident concerning the role of the 5-HT₃ receptor in anxiety and a possible interactional model has been investigated in some studies (Andrews et al, 1994).

1.3.4.5 The 5-HT₅ Receptor

5-HT₅ receptors have been isolated from mice (Hen et al, 1992) and rat brain c-DNA libraries (Wisden et al, 1993) in A and B forms. A human 5-HT_{5A} receptor has also been cloned (Rees et al, 1994) but no evidence is currently available for these receptors in the natural state. It is likely that 5-HT_{5A} comprises 357 amino acids while 5-HT_{5B} may have approximately 370 amino acids.

The 5-HT_{5A} human receptor gene has been located on chromosome 7 at position 7q 36 and the 5-HT_{5B} human receptor gene on chromosome 2 at 2q11-13, however it is not clear if this receptor is expressed (Rees et al, 1994). The receptor would appear to have a widespread brain distribution, according to in-situ hybridisation studies (Waeber et al, 1998).

In mice, the regions linked with 5-HT_{5A} m-RNA are neurones within the cerebral cortex, dentate gyrus and the hippocampal CA1-3 pyramidal layers, cerebellum granule layer and the olfactory bulb (Plassat et al, 1992). Similar studies have shown the B receptor m-RNA in the hypothalamus, hippocampus, medial and lateral habenula, DRN, olfactory bulb, and the entorhinal and pyriform cortices (Erlander et al, 1993). These are at low intensity and post-natal timing studies also show different rate of expression, between the two m-RNA species. Human brain tissue also exhibits increased translation in adults, and astrocytes are a major expression site early in post-natal life. 5-HT_{5B} is late in expression but appears shortly after birth in the rat (Wisden et al, 1993).

In its pharmacology 5-HT₅ is similar to 5-HT₄. As with many other 5-HT receptors, 5-HT receptors are G-protein coupled receptors with 7 transmembrane domains. The receptor appears to reduce production of forskolin stimulated adenylate cyclase, (Francken et al, 1998a and b). In knock-out mice increased locomotion has been seen and exploratory behaviour, but this did not seem to be related to anxiety features on animal testing (Grailhe et al, 1997).

1.3.4.6 The 5-HT₆ Receptor

In common with the other 5-HT receptors, the 5-HT₆ receptor is a member of the G-protein-coupled, seven putative transmembrane domain, receptor superfamily. It was detected by the technique of nucleotide sequence homology screening, by two groups (Ruat et al, 1993; Monsma et al, 1993).

Agonist induced desensitisation has been described, probably caused by c-AMP-dependant protein kinase, leading to receptor phosphorylation. The receptor gene is located on chromosome 1 at 1p35-36. In the rat, high levels of receptor m-RNA are only found in the central nervous system, especially in the caudate nucleus (Monsma et al, 1993).

High levels of 5-HT₆ receptors have been located in the hippocampus, olfactory tubercles and nucleus accumbens, and immunocytochemistry has concurred with these regions as well as labelling the cerebral cortex and striatum (Gerard et al, 1997). In spite of the difficulty localising the receptors, due to low levels of expression and the high rate of non-specific binding of ligands, studies have supported a post synaptic position of the receptors. This has been confirmed by the absence of loss of raphe 5-HT₆ receptor m-RNA after neuronal lesions. It has also been proposed that localisation occurs on dendrites of hippocampal and striatal neurones as well as on GABA-ergic neurones terminating in the substantia nigra and globus pallidus (Gerard et al, 1997).

From a psychiatric perspective, interest in the psychopharmacology of the receptor has centred around the capacity of antipsychotics, including clozapine, to antagonise the receptor and a further study has used the radioligand (³H) Clozapine, in an additional attempt to characterise the receptor, (Glatt et al, 1995). Other drugs are also now being evaluated, with known effect on the receptor as a target (Branchek et al, 2000; Bromidge et al, 2001). Both native and recombinant 5-HT₆ receptors have been shown to positively enhance adenylate cyclase (Boess et al, 1997).

Behavioural effects of the antagonism of the 5-HT₆ receptor have included rat 'yawning and stretching' following I.C.V. antisense injection in [3H] LSD sites (not D₂ or 5-HT₂), (Bourson et al, 1995). This finding has been further supported by a different group and prefrontal 5-HT release was attenuated (Yoshioka et al, 1998).

Knock-out mice have shown increased anxiety in animal models. In studies where levels of corticosterone are artificially adjusted, 5-HT₆ receptor m-RNA is elevated with corticosteroid absence. This may be relevant to the vulnerability of some people to depression (Boess et al, 1997).

1.3.4.7 The 5-HT₇ Receptor

This most recently identified serotonin receptor seems to be an equivalent of the fruitfly (*Drosophila Melanogaster*) 5-HT_{dro1} receptor (Witz et al, 1990) and is between 445 and 448 amino-acids in length. Its gene, containing 2 introns, is located on chromosome 10 (q21-24) in humans (Gelernter, 1995). The receptor exhibits features common to the G protein-coupled receptor superfamily.

While 4 receptor splice variants exist (q-d), 5-HT_{7(d)} is absent in rat tissues and 5-HT_{7(c)} has not been detected in human tissue, (Heidmann et al, 1997). The 5-HT₇ receptor is found in higher concentrations in the hippocampus, thalamus and hypothalamus than in the cortex and amygdala (Gustafson et al, 1996).

The 5-HT₇ receptor is also affected by anti-psychotic and anti-depressant drugs but the gene has not been implicated in vulnerability to either schizophrenia or bipolar disorder (Erdmann et al, 1996). The receptor transduction system involves stimulation of adenylate cyclase.

From a functional perspective, it would seem that suprachiasmatic nucleus activity and phase shifting potential support a role for the receptor in circadian rhythms. 5-HT₇ mRNA has been located in the SCN and cAMP also induces a phase shift, as is seen with 5-HT (Prosser and Gillette, 1989).

One possible neuropsychiatric role for the receptor would be in the reduction of fits in epilepsy for which hypothesis animal models exist (Bourson et al, 1997). In anti-sense experiments with rats, an anxiety paradigm was not supported (Clemett et al, 1998).

Fluoxetine has been shown to cause down regulation in 5-HT₇ receptors over time (Sleight et al, 1995) but other SSRIs were not found to affect 5-HT₇ receptor mediated stimulation of cAMP levels. However, desensitisation was seen after direct receptor stimulation (Shimuzu, 1998). Some tricyclic drugs have been shown to enhance 5-HT stimulated cAMP production.

1.4 DEPRESSION AND 5-HT

1.4.1 Measures of brain 5-HT function

1.4.1.1 CSF 5-HIAA

Following on from knowledge of the metabolic cycle of serotonin production and degradation, it is apparent that measuring the quantity of breakdown products of 5-HT may give an estimation of the total activity of serotonin at earlier stages in the cycle. In order to closely assess serotonin activity within the central nervous system studies have looked at the level of 5-HIAA in the cerebro-spinal fluid withdrawn from the subarachnoid space by the clinical procedure of lumbar puncture.

Studies have generally not shown reduced levels in depressed patients (Koslow et al, 1983) , but some studies have shown decreased levels in patients with a history of suicide attempts (Brown and Linnoila, 1990). It has also been reported that this correlation is greater where the attempts to self harm have been particularly violent (Mann and Malone, 1996). It is of note that similar low CSF 5-HIAA levels have been found in patients diagnosed with personality disorders, chiefly dissocial personality disorder, where violent and aggressive behaviours have been evidenced (Linnoila and Virkunen, 1992) leading to the theory that the low levels may be markers for personality traits associated with violence and anti-social expression and these traits modify behavioural presentations in variable mood states (Roy et al, 1990).

It has also been clarified in the latter reference that the finding of low 5-HIAA levels is not diagnosis specific and some studies have shown similar low levels in schizophrenia.

1.4.1.2 Post-mortem binding studies

A further prediction of the above discussion is that levels of 5-HT receptor binding should be reduced in the brains of those dying by means of suicide. Studies have not provided an unequivocal answer, but it would presently seem that the evidence is not consistent for reduced levels (Horton, 1992). However, as discussed above, there is more consistent evidence that those depressed patients attempting to commit suicide have lower levels of CSF 5-HIAA. As this finding is not only seen in depressives following suicide attempts but also in some samples of schizophrenic patients and those with personality disorder where there is a history of aggression, it has been suggested that the finding in suicides may also correlate less with mood and more with a tendency to react impulsively in certain situations (Brown and Linnoila, 1990).

Attempts have also been made to establish whether variations in brain 5-HT or 5-HIAA are abnormal post suicide as a more direct test of the mono-amine hypothesis. Interpretation of results of these studies is potentially complicated by factors including – brain changes which may have occurred following death, and changes which may have occurred before death but due to factors other than depression eg: anoxia or due to drugs taken therapeutically or by over-dose. An early series of studies evaluated Tritiated imipramine binding in suicide victims, as a measure of serotonin transporter number.

Although the initial studies showed a reduction in cortical binding (Stanley et al, 1982; Perry et al, 1983), a later study was unable to replicate the finding (Horton, 1992). More recently, Lawrence was also unable to replicate the original finding (Lawrence et al,1998), and agreed with the Horton study, with a general current perspective that levels of 5-HT and 5-HIAA are not consistently reduced.

Receptor subtypes examined in post-mortem tissue other than the serotonin transporter include 5-HT₁ and 5-HT₂ subtypes. The 5-HT_{1A} receptor numbers seen in brains of suicide victims do not show changes in a specific direction (Horton, 1992; Arranz et al, 1994). Lowther has looked at both this receptor (Lowther et al, 1997a) and other 5-HT₁ receptors (Lowther et al,1997b). An increase in pallidal 5-HT_{1D} receptors was found in the drug-naïve depressive group but the 5-HT_{1E} and 5-HT_{1F} receptor numbers were unchanged.

Some receptor studies revealed an increase in the number of 5-HT₂ binding sites in the frontal cortices of suicide victims (Mann et al, 1986) however, although initially reported as correlating especially well in those dying by violent means (Arora et al, 1989), a more recent extensive study did not show this (Lowther et al, 1994). Most recently, the brain scanning method SPET has been used to assess receptor function in serotonergic drug naïve suicide attempters. In one study involving a 5-HT_{2A} antagonist, deliberate self harmers had significantly reduced measures of frontal binding than controls. This effect was also more pronounced in self injury than self-poisoning (Audenaert et al, 2001).

It is also not clear that severe depression would be the only mediating factor towards upregulation of 5-HT₂ receptors as a similar finding was reported in the brains of those dying of other causes than suicide (Yates et al, 1990), and dieting has led to an increase in the sensitivity of one receptor subtype, the 5-HT_{2C} receptor (Cowen et al, 1996).

Drug therapy is also known to affect 5-HT_{2A} numbers. A reduction of 5-HT_{2A} receptors has been seen with chronic treatment using tri-cyclic anti-depressants (Cowen, 1990) but this is not the effect seen, after similar treatment periods, on platelet 5-HT_{2A} receptor numbers, which are increased.

1.4.1.3 Neuro-endocrine studies

The tests described in this section relate to attempts to use hormonal measures to index the activity of 5-HT receptors. Probes that increase 5-HT function increase the secretion of anterior pituitary hormones by facilitating the release of peptide releasing hormones in the hypothalamus.

As can be predicted by an understanding of the cellular systems for the production and metabolism of 5-HT and related aspects of neuro-transmission, probes exist which relate to both pre-synaptic neuronal function and to direct effects on post-synaptic receptors.

Responses to Tryptophan:

In normal subjects plasma hormonal responses to intra-venous tryptophan (TRP), the 5-HT precursor, include elevations of prolactin and growth hormone. As discussed previously, these responses can be attenuated by the administration of pindolol but not by 5-HT₂ or 5-HT₃ receptor antagonists, implying that 5-HT_{1A} antagonism is causing the attenuation of the response (Cowen, 1993). Endocrine responses to TRP in depressed patients are blunted in most of the relevant studies. In some of these studies changes in body weight or TRP availability have to be considered in interpretation of the findings.

Thus in two studies (Cowen et al, 1987; Deakin et al, 1990), significant blunting of the hormonal responses was only seen when those patients with significant recent weight loss were excluded, and this has been recognised as a confounding variable in diet studies where weight loss has led to an increased PRL response to TRP. In another study the plasma levels of TRP were low, implying that this might be the reason for a reduced response, however five studies have reported blunted PRL and GH responses of depressives to TRP in the absence of changes in TRP availability (Reviewed in Power, 1992).

In order to establish whether these attenuated responses might reflect a trait abnormality, one study examined neuro-endocrine responses to TRP once patients had recovered from the depressive episode and been withdrawn from treatment. The results suggested that abnormal endocrine responses to TRP normalised with clinical recovery (Upadhyaya et al, 1991).

Responses to 5-HTP

An increase in plasma cortisol is seen following the administration of 5-HTP (Gartside and Cowen, 1990). As this response is not attenuated by pindolol, it is unlikely that the 5-HT_{1A} receptors are involved in the response, however the response is blunted by ritanserin, suggesting mediation via indirect activation of 5-HT₂ receptors is the underlying mechanism (Meltzer and Maes, 1994). Results from studies in depressed patients are contradictory but an increased response may be present in depressed women. Whether this could be due to concomitant weight loss is unclear.

Responses to d-Fenfluramine

d-Fenfluramine is an amphetamine analogue which promotes the release of 5-HT from terminal vesicles and also functions as a serotonin re-uptake inhibitor. The prolactin responses to d-Fenfluramine are probably mediated via indirect activation of 5-HT₂ receptors as the PRL response is blocked by ritanserin but not pindolol (Park et al, 1995). The origin of the cortisol response is less clear.

Some studies have reported blunted responses to d-Fenfluramine in depression but this has not been seen consistently. Four studies have reported attenuated responses but these have included specific subtypes by either severity of mood (Mitchell et al, 1990; Lichtenberg et al, 1992) or personality disorder (Coccaro et al, 1989; Malone et al, 1996) where blunted responses have also been reported, in the absence of low mood (Stein et al, 1996).

This latter effect has been further investigated by deriving separate measures of the personality traits psychoticism and aggression. d-Fenfluramine induced cortisol non-response occurred to a greater extent in those with higher psychoticism scores while PRL blunting was more evident in those with aggressive traits (Netter et al, 1999). In a study involving offenders, psychopathy correlated with reduced initial cortisol responses while non-psychopathy and schizoid personality were associated with enhanced PRL response. Reduced 5-HT function was reported in those with borderline disorder, recurrent self-harm or alcoholism (Dolan et al, 2001). There is some evidence that the PRL response to d-Fenfluramine is a trait marker of depression (Reviewed in Lerer et al, 1996).

d-FEN has also been used to test the Cloninger model of tri-dimensional personality types. In the severely depressed patient group mentioned above, the blunted responses have been investigated in a similar way to those following TRP, once the person has recovered. Likewise, in two studies including intra- and post-illness testing, normalisation was seen following clinical recovery, however in one study with a more extended follow-up period of one year, patients had stopped their medication by the time of the second test and blunting again returned, suggesting a possible return to a vulnerable state, but without clinical relapse (Gerra et al, 2000). In a further investigation of the relationship between 5-HT release, hormonal responses and mood, d-fenfluramine was administered to patients with unipolar major depression. Baseline cortisol measures correlated inversely with severity but higher initial cortisol responses predicted better therapeutic response (Cleare et al, 1998). It has also been shown that pindolol can block prolactin but not cortisol release after d-fenfluramine administration (Palazidou et al, 1995).

Unfortunately, risks are now recognised to be associated with fenfluramine use and indices of neurotoxicity, pulmonary hypertension and cardiac valve pathology have been reported. Direct toxic effects on the serotonin transporter have been described using a human placental cell model involving DNA fragmentation and apoptosis but this effect was blocked by the use of the SSRI, Fluoxetine, suggesting that intact 5-HTT function is necessary for apoptosis to occur (Bengel et al, 1998). However, other data suggests that d-FEN induced valvular heart disease only occurs with the involvement of 5-HT_{2B} agonists, implying a complex toxicity model (Rothman, 2000).

Responses to clomipramine:

All tricyclic anti-depressants exhibit a combination of serotonergic and noradrenergic activity but Clomipramine has been widely used as a probe of 5-HT function as it is the tricyclic antidepressant with the most significant level of serotonergic activity by re-uptake inhibition and relatively low levels of noradrenergic activity. By using this drug as a probe it is possible to use prolactin and cortisol as proxy markers of central 5-HT function, as described above (Cowen, 1993). Chronic treatment with clomipramine has been shown to lead to down-regulation of 5-HT₂ receptors and a possible serotonin/ dopamine interaction using PET (Attar-levy et al, 1999). The major metabolite of clomipramine is desmethylclomipramine, which is a potent inhibitor of noradrenaline re-uptake but is not detectable within the testing period following administration of intra-venous clomipramine and is therefore not thought to interfere with interpretation of the outcomes of the clomipramine challenge as a measure of 5-HT activity.

It has been reported that the prolactin response to *i/v* clomipramine is abolished by the non-selective 5-HT receptor antagonist, methysergide, suggesting that post synaptic receptors are involved in the response. Unfortunately doses high enough to cause significant hormonal responses are often associated with nausea and vomiting, limiting the use of the challenge. Three studies in depressed patients have found blunted PRL responses to clomipramine, in comparison with healthy controls (Reviewed in Cowen, 1998).

5-HT receptor agonists

There are a number of drugs which have been studied in view their high affinity for the 5-HT_{1A} receptor, including buspirone, gepirone, ipsapirone and tandospirone. Although administration of buspirone, the most widely studied, increases plasma ACTH, PRL, GH and cortisol as well as eliciting a hypothermic response in most studies, elucidation of the specific mechanism has been complicated by additional effects on the dopamine system. As the effects common to the listed 5-HT_{1A} agonists include GH and ACTH release and hypothermia it has been possible to use pindolol to attenuate these responses (Reviewed in Cowen, 1993). PRL responses to the probes are inconsistent.

A further 5-HT_{1A} agonist, 8-OH-DPAT has been used in animal studies where reproduction of the above effects by direct injection into the hypothalamus has supported previous evidence that the endocrine responses are due to post-synaptic 5-HT_{1A} receptors (van der Kar, 1991).

However, direct injection of 8-OH-DPAT into the DRN causes hypothermia, suggesting that this response is mediated by autoreceptors on the cell bodies of 5-HT_{1A} neurones, as ligand binding studies have shown that these receptors are located on both cell bodies and post-synaptically in the hypothalamus.

The relatively selective 5-HT_{2C} receptor agonist m-CPP will be discussed in detail below. A further receptor agonist acting on this receptor is MK212, which also affects 5-HT_{2A} receptors but has a similar hormonal response profile to m-CPP. Both 5-HT_{1A} agonists and 5-HT_{2C} agonists therefore increase ACTH and cortisol but increases in GH are seen to a greater extent in 5-HT_{1A} activation. PRL elevation is more reliably caused by 5-HT_{2C} activation which also causes hyperthermic responses while 5-HT_{1A} activation leads to a hypothermic reaction (Reviewed in Cowen, 1998). The above agonists are the most widely investigated probes of function however newer, more highly selective agonists and antagonists are also being studied, as discussed in the sections above, describing the individual receptors.

1.4.1.4 Tryptophan studies

Plasma studies

In view of the competition between TRP and other amino acids for entry to the brain, it was previously hypothesised that a correlation should exist between brain TRP and both plasma TRP and the relative ratio of TRP to the other large neutral amino acids. One animal study showed that 5-HT synthesis increased following an increase in available peripheral TRP (Gibbons et al, 1979).

A corollary of the hypothesis that optimal serotonin activity is necessary to protect individuals against depression is that plasma levels of TRP, if reflecting brain tryptophan activity, should be lower in depressed patients. A number of studies have investigated this corollary with variable results. One study looking at serum 5-HT itself only showed a reduction in the plasma of the melancholic subgroup of depressed patients (Perez et al, 1998). The most consistent reductions are seen in total plasma tryptophan both in the latter study and others (De Myer et al, 1981; Karege et al, 1994; Anderson et al, 1990; Malatino et al, 1982) and the TRP: amino acid ratio (Anderson et al, 1990; De Myer et al, 1981; Cowen et al, 1989). However, no change was seen in total TRP levels in some studies (Niskanen et al, 1976; Moller et al, 1979; Perez et al, 1998; Perret et al, 2000). Total TRP was actually increased in daytime measures in depressives in the circadian study (Malatino et al, 1982), a finding also seen for serum 5-HT in the summer (Sarrias et al, 1989). Results have been equivocal for free plasma tryptophan with a reduction in depressives (Cowen et al, 1989), an increase in depressives (Niskanen et al, 1976) or no difference in comparison with normals reported (Moller et al, 1979).

Tryptophan depletion studies

As TRP is a precursor for serotonin it has been hypothesised, as a corollary to the evidence linking 5-HT and depression, that experimental manipulation of TRP levels in the body might contribute to a lowering of mood. Previous animal studies have shown that peripheral TRP depletion leads to a reduction in available TRP for central synthesis (Biggio et al, 1974).

This has been investigated in healthy individuals with both positive (Smith et al, 1997a and b; Young et al, 1985; Ellenbogen et al, 1996) and negative results (Oldman et al, 1994; Abbott et al, 1992). In one study a deterioration in mood only occurred in those patients with a positive family history of affective disorder (Benkelfat et al, 1994) while in another study the effect was confined to females (Smith et al, 1997a). In depressive patients tryptophan depletion has also been shown to lower mood, during periods of remission (Smith, 1997b).

The technique - 'Tryptophan depletion', is achieved by subjects drinking an amino-acid rich drink which lacks TRP. There is evidence that this technique lowers the concentration of tryptophan in plasma (Delgado et al, 1990), and inhibits transport across the blood-brain barrier (Biggio et al, 1974; Young et al, 1985). Two physiological means by which the technique might achieve this are through the production of protein in the liver, which could be stimulated by the amino-acid drink, and subsequent uptake of existing stores of TRP in the plasma or through competition of the other amino acids for transport across the blood-brain barrier. It is likely that both mechanisms play a part in reducing TRP availability to the brain.

1.4.1.5 Platelet studies

Platelet 5-HT has been shown to have either consistent levels between normals and depressives (Perez et al, 1998; Karege et al, 1994) or a reduction in depressives (Quintana et al, 1992).

As human platelets were found to express a functional 5-HT receptor (Geaney et al, 1984), which resembled pharmacologically the 5-HT_{2A} receptor in the brain, it was hoped that peripheral measures of 5-HT 5-HT_{2A} receptor binding might relate in a direct manner to brain 5-HT_{2A} receptor function.

In addition to the 5-HT₂-like receptor, thought to promote platelet aggregation, there is also a binding site, to which tritiated forms of imipramine and paroxetine bind, which may be part of the 5-HT transporter (Nemeroff et al, 1994). However, it is not clear that peripheral measures of 5-HT binding can be used to estimate brain activity as one study comparing the effects of central 5-HT lesions in rats on levels of 5-HT and receptor binding showed significant brain changes but peripheral changes were not observed in the paroxetine binding (Moret et al, 1991).

In the following section attention is focused more specifically on the 5-HT_{2C} receptor and the most widely used agonist used in the investigation of its action. As has been seen above, the 5-HT_{2C} receptor is influenced significantly by some anti-depressants and in view of further studies (See below) linking one of the polymorphic variants of its gene with response to clozapine, it is important that due consideration is given to a possible key role in drug treatments for both depression and the psychoses.

1.5 5-HT_{2C} RECEPTOR FUNCTION

In this section relevant studies relating to the function of the 5-HT_{2C} receptor are reviewed, including investigations reported from animal studies, healthy human controls and psychiatric patients including a broader range of diagnoses than depression and the psychoses.

1.5.1 Animal studies

The majority of studies investigating the role of 5-HT_{2C} receptors have utilized meta-chlorophenylpiperazine(m-CPP) as a neuroendocrine probe (See Figure 1.3). m-CPP is a 5-HT agonist whose pharmacological effects have been extensively studied in both humans and animals. According to ligand-binding studies m-CPP binds to a variety of 5-HT receptors but its highest affinity is for the 5-HT_{2C} receptor (Hoyer, 1998).

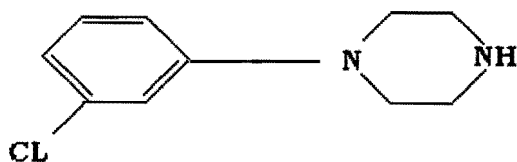


Figure 1.3: Diagrammatic representation of the 5-HT_{2C} selective agonist, meta-Chlorophenyl-piperazine.

1.5.1.1 Locomotor effects:

Decreases in locomotor activity in rats is a characteristic behavioural effect of m-CPP. Studies with selective antagonists suggest that the hypolocomotor effects are produced by activation of 5-HT_{2C} receptors because they are antagonised by a variety of drugs that have in common the ability to block 5-HT_{2C} receptors. (Kennedy et al, 1993; Kennet and Curzon, 1988; Klodzinska, 1989). The locomotor effect is also blocked by less selective compounds such as metergoline, methysergide, and mianserin pre-treatment (Lucki et al, 1989). The locomotor suppressant effects of m-CPP have been enhanced following long term, but not short term, treatment with imipramine (Aulakh et al, 1987). In animal studies the hypolocomotor response was enhanced by intraventricular injection compared to intra-peritoneal injection, suggesting a central site of action (Kennet and Curzon, 1988).

1.5.1.2 Appetite and weight

In animal studies m-CPP has consistently lowered food intake (Samanin et al, 1979; Kennett and Curzon, 1988 and 1991), probably by activation of the 5-HT_{2C} receptors in the paraventricular nucleus (Aulakh et al , 1995). m-CPP induced hypophagia has also been reversed by the 5-HT antagonists - metergoline, mianserin and mesulergine but not ritanserin, in one study (Aulakh et al, 1992). The negative finding with ritanserin was replicated, along with ketanserin, in a further study (Kennet and Curzon, 1988).

However, the hypophagic effects were inhibited by ketanserin and ritanserin in a subsequent study by the same authors (Kennet and Curzon, 1991). Hypophagic effects were enhanced in an earlier study following long term treatment with imipramine (Aulakh et al, 1987). Further work has suggested that gender differences in the hypophagic response to m-CPP may occur (Clifton et al, 1993).

As a test of the above model one study (Tecott et al, 1995) has reported the behavioural and morphological changes resulting from the genetically engineered, 'knock-out' mice lacking 5-HT_{2C} receptors. In keeping with the above studies establishing the relevance of the 5-HT_{2C} receptor to appetite and weight, these mice were significantly overweight and displayed other differences such as a lower seizure threshold. Further studies involving these knock-out mice established that their obesity was behavioural rather than metabolic and that hypophagic effects of m-CPP were abolished in neuro-endocrine testing.

1.5.1.3 Neuro-endocrine effects:

Intra-venous administration of m-CPP to rats has increased plasma prolactin and corticosterone levels in some studies (Aulakh et al, 1988; Fuller et al, 1981; Quattrone et al, 1981; Bagdy et al, 1989), however antagonist studies have shown attenuation of prolactin release by metergoline but not ritanserin in rats and only partial blockade by ritanserin in Rhesus monkeys (Kahn and Wetzler, 1991).

ACTH/corticosterone is increased by m-CPP in rats and Rhesus monkeys (Fuller et al, 1981; Bagdy et al 1989; Aulakh et al, 1988; Calogero, 1990). Metergoline and ritanserin, but not ketanserin, attenuated ACTH release but corticosterone was not blocked by metergoline in one study (Aloi et al, 1984) which may be explained by the intrinsic ability of the antagonists to increase corticosterone themselves (Aulakh et al, 1992). m-CPP has also been shown to increase growth hormone levels in Rhesus Monkeys but to decrease levels in rats (Aulakh et al, 1993). Long term administration of m-CPP has been shown not to affect prolactin and corticosterone release (Ulrichsen et al, 1992). In one study involving rats, high doses of intra-venous m-CPP caused increases in adrenaline and noradrenaline in plasma (Bagdy et al, 1988).

1.5.1.4 Sleep

Early animal work established a central role for serotonin, and other amines, in the regulation of sleep and wakefulness (Sallanon, 1983). In hamsters 5-HTP increased total sleep time and methysergide facilitated REM sleep but inhibited non-REM sleep (Guha et al, 1988). In rats, decreases in REM sleep caused by destruction of 5-HT neurons is reversed by the transplant of foetal raphe suspensions (McRae et al '88).

Anti-depressants have variable effects on sleep and SSRI induced increases and decreases in slow wave sleep have both been reported (Hilakivi, 1987), but most reduce REM sleep volume.

An early study involving mice showed the prolongation of thiopentone induced sleep by both Trazodone and m-CPP, an effect which was not prevented by cyproheptadine (Adamus et al, 1985). However, studies in rats have shown a reduction in slow wave sleep due to m-CPP (Dugovic and Van den Broek, 1991).

Another drug with 5-HT₂ agonist properties, DOI, also decreases slow wave sleep (Stutzmann et al, 1992). In addition, increases in slow wave sleep are seen with selective 5-HT₂ antagonists (Dugovic and Waquier, 1987; Stutzmann, 1992).

1.5.1.5 Temperature

Intraperitoneal administration of m-CPP has been shown to cause a hyperthermic response with a peak effect at thirty minutes (Mazzola et al, 1996). This effect is thought to be mediated by 5-HT_{2C} receptors as it is attenuated by pre-treatment with low-dose metergoline, mesulergine and mianserin but not propranolol, yohimbine or ondansetron.

High doses of ketanserin, ritanserin and spiperone have also attenuated the hyperthermic responses to m-CPP and both acute and chronic treatment with MAOI's and tri-cyclic antidepressants has also been shown to attenuate this response (Wozniak et al, 1989).

Interestingly, in mice temperature is decreased by m-CPP.

1.5.1.6 Anxiogenic effects

The anxiogenic effect of m-CPP has been recorded by a number of investigators, in humans (Charney et al,1987; Mueller et al, 1985). This effect has been subsequently substantiated in male rats by a model involving decreased social interaction and activity, using m-CPP and TFMPP (Kennett et al, 1989) and is thought to be mediated by hippocampal 5-HT_{2C} receptors. This finding was further corroborated by studies involving 5-HT_{2C} antagonists (Kennett et al, 1989). Support for this paradigm representing a model of anxiogenesis has also been found with the prevention of the effect by a benzodiazepine (Tyers et al, 1989) .

1.5.2 Human studies – m-CPP use in healthy subjects

m-CPP, a metabolite of the anti-depressants trazodone and nefazodone, has also been extensively studied in healthy humans. It has been found to be one of the more reliable probes with which to measure aspects of brain 5-HT function although debate continues regarding its specificity. It is a 5-HT_{2C} and possibly 5-HT_{1B} agonist but is a 5-HT_{2A} antagonist (Kahn and Wetzler, 1991; Fiorella et al, 1995). It also binds weakly to 5-HT_{1A}, 5-HT_{1D}, and 5-HT₃ receptors and also has α_2 adrenergic activity. It has a weak effect on α_1 and β adrenergic receptors and binds very weakly to dopamine and muscarinic receptors (Hamik and Peroutka, 1989). It is a partial agonist at 5-HT_{2C} and 5-HT_{1B} receptors and may release 5-HT from the pre-synaptic terminal (Baumann et al, 1993).

The studies involving animals have established the basic effects of m-CPP and subsequent work has examined whether the findings can be extrapolated to humans. The effect of m-CPP on temperature, appetite, sleep and neuroendocrine measures has therefore been studied in healthy humans for more than a decade (Mueller et al, 1985).

1.5.2.1 The effect of m-CPP on temperature

As in animal studies, m-CPP has been found to increase temperature after oral (0.5 mg/kg) and intravenous (0.1mg/kg) administration (Mueller et al, 1985; Murphy et al, 1989) in most studies, however Kahn and colleagues failed to find a hyperthermic effect (1990).

1.5.2.2 The effect of m-CPP on sleep

The possibility that 5-HT₂ receptors may inhibit slow wave sleep (Stages 3 and 4, < 5Hz) in humans was suggested by an increase in slow wave sleep following acute and chronic ritanserin treatment (Idzikowski et al, 1986/7; Sharpley et al, 1990).

In an initial report on six normal subjects m-CPP reduced total sleep time and sleep efficiency and decreases in slow wave sleep and REM sleep were found (Lawlor et al, 1991). Furthermore, stage 1 sleep was prolonged. This finding was replicated at two doses against placebo (7,5 mg and 15mg) with a significant reduction in slow wave sleep and the number of REM periods (Katsuda et al., 1993).

Further studies with the 5-HT₂ receptor antagonists ritanserin and ketanserin have established that this effect is almost entirely a 5-HT_{2C} response as both have equivalent affinities for the 5-HT_{2A} receptor but ritanserin has a higher affinity for the 5-HT_{2C} receptor than ketanserin and produced a substantially larger increase of slow wave sleep, 51,4 % versus 17,2 and 24,4% (Sharpley et al, 1994). In an effort to establish whether this effect would diminish following SSRI treatment, 8 depressed patients were compared with 8 controls with no difference seen between the groups, suggesting that no reduction in 5-HT_{2C} sensitivity had occurred (Williams et al, 1994), at least as far as this measure was concerned.

1.5.2.3 The effect of m-CPP on appetite and weight

The involvement of the 5-HT system in appetite has been well established in animals such as the rat (above) and the leech, where serotonergic neurons regulate the cycle of arousal, food seeking, ingestion and satiation (Lent and Dickenson, 1988). In humans, food intake was not reduced following m-CPP given in a standard oral dose regimen (7.5 or 15 mg)(Smith et al, 1994).

Subsequently, using a dose calculated according to weight (0.4mg/kg), food intake in a test meal has been shown to be reduced in both males and females following the administration of m-CPP to 12 healthy volunteers (Walsh et al, 1994). This was further replicated in 24 subjects (Cowen et al, 1995).

Furthermore, the fact that drugs such as olanzapine and mianserin which block 5-HT_{2C} receptors, cause troublesome weight gain in clinical use supports the notion that 5-HT_{2C} receptors play a role in food intake in humans. The effect on weight is supported by weight gain in patients on drugs such as mianserin which also block these receptors.

1.5.2.4 Neuroendocrine findings

m-CPP has been used extensively as a neuro-endocrine probe in humans and reliably elevates plasma levels of prolactin, cortisol and ACTH (Kahn and Wetzler, 1991). Peak ACTH levels were shown to precede elevation of cortisol levels in one study, suggesting causation (Kahn et al, 1990). Intravenous m-CPP, but not an oral dose, has been noted to increase growth hormone levels (Cowen , 1993) as well as causing physiological effects such as light-headedness, increased blood pressure in some subjects or headache (in approx.50% of people), as well as panic in 10-30% of people (Silverstone et al, 1994).

The exact control mechanism of prolactin has been extensively studied and it is clear that both stimulatory and inhibitory transmitters play a role. However there is evidence that m-CPP induced prolactin release is a reliable measure of 5-HT₂ function as the increase is attenuated by pre-treatment with the 5-HT₂ antagonists metergoline and ritanserin. This attenuation is not found with the growth hormone response. Because mCPP is a 5-HT_{2A} receptor antagonist its effects on prolactin and cortisol are likely to be mediated via activation of 5-HT_{2C} receptors (Hamik and Peroutka, 1989). Gender differences in responses are seen with a greater female prolactin but not cortisol response (Charney et al, 1987; Kahn et al, 1990).

1.5.3 Human studies – m-CPP use in psychiatric patients

1.5.3.1 Depression

As previously reviewed (Kahn and Wetzler, 1991), when given to patients with major depression, m-CPP was found to cause more physical symptoms than in the healthy controls, but without other behavioural differences. Hormonal responses such as cortisol and prolactin did not differ from controls and the blood levels of m-CPP in the two groups were not significantly different either.

A seasonal variation in behavioural responses to m-CPP has been found in patients suffering from seasonal affective disorder when compared to controls (Joseph-Vanderpool et al, 1993). m-CPP has also been studied as a therapeutic agent and was found to be mildly anti-depressant when 80mg/day was given for two weeks (Mellow et al, 1990).

1.5.3.2 Schizophrenia

While the original studies evaluating the effect of m-CPP on the symptoms of schizophrenic patients produced equivocal results, some suggesting a blunting of behavioural and hormonal responses and others an enhancement, a later study has shown an exacerbation of positive symptoms, particularly the BPRS thought disorder factor, in the patient group but not in controls (Krystal et al, 1993).

This finding, supporting a role for 5-HT_{2C} receptors in schizophrenia, is further strengthened by the correlation of positive responses to m-CPP challenge prior to treatment with likelihood of treatment response to Clozapine (Kahn et al, 1993). In addition Clozapine has been found to block responses to m-CPP while fluphenazine had no effect (Owen et al, 1993).

1.5.3.3 Obsessive Compulsive Disorder

Studies reviewed previously (Kahn and Wetzler, 1991) suggested that an oral dose of m-CPP (0.5 mg/kg) increased obsessions and caused more anxiety in obsessive compulsive disorder (OCD) patients than in normal controls. This symptomatic worsening was abolished by treatment with clomipramine. In one replication (Pigott et al, 1992), intravenous m-CPP (0.1mg/kg) increased OCD symptoms and anxiety ratings, an effect blocked by metergoline. The m-CPP was given as a 90 second bolus compared to a previous study where intravenous administration occurred as an infusion over 20 minutes and no increase in OCD symptoms was seen (Charney et al, 1988).

In a further study peak m-CPP aggravated symptoms in 55% of the OCD patients but the increased behavioural measures correlated with less effect on prolactin release, suggesting a complex system involving numerous neuro-transmitter and neuro-modulatory effects. (Hollander et al, 1992). In another attempted replication, (Goodman et al, 1995) no exacerbation of OCD symptoms was seen but both i.v. and oral m-CPP caused anxiety.

1.5.3.4 Anxiety and panic

The anxiogenic effect of m-CPP seen in rats has been noted in human studies.

Although an original study reported no behavioural differences between panic disorder patients and controls to an m-CPP challenge (Charney et al, 1987), a subsequent study showed increased anxiety and cortisol responses in a group of panic subjects compared with normal subjects and depressives (Kahn et al, 1988). This was further corroborated by the finding of a subject with sub-clinical panic attacks who displayed exaggerated responses to m-CPP (Kalus et al, 1990).

In a sample of 10 patients with generalised anxiety disorder a greater behavioural response to m-CPP was seen than in healthy subjects, as well as an anger response (Germine et al, 1992). More recently repeated administration of m-CPP has been found to attenuate behavioural responses, such as anxiety, to an m-CPP challenge (Benjamin et al, 1996).

The role of the serotonergic system in anxiety has been further supported by the association of a serotonin transporter gene polymorphism with anxiety related personality traits in individuals as well as in sibships (Lesch et al, 1996; Goldman, 1995).

1.5.3.5 Eating Disorders

In one study of the effect of m-CPP on bulimic patients (Brewerton et al, 1992), a number of the patients developed migraine headaches. There would thus appear to be a correlation between the eating disorder and a tendency to develop migraines.

This was not only the case where the bulimic patients had a personal history of migraine but was also seen in those with a family history of migraine. Although it could be predicted that patients with either bulimia nervosa or binge eating disorder would have a higher rate of the less common serine substituted 5-HT_{2C} polymorphism than the general female population, this was not shown in one Oxford study (Burnet et al, 1999).

The work of Tecott and colleagues (1998) also has relevance as the 5-HT_{2C} receptors would appear to be needed to evoke the satiety response in feeding, as 5-HT_{2C} knock-out mice become obese.

The neuro-endocrine results of the studies involving bulimic patients have shown a blunted prolactin response. In a further study anorectic patients were seen to exhibit a greater behavioural response to m-CPP prior to weight gain and diminished neuroendocrine responses. After weight gain the prolactin response remained reduced (Hadigan et al, 1995) suggesting a trait subsensitivity of hypothalamic 5-HT_{2C} receptors. Conceivably this could be related to a tendency to binge-eat.

1.6 5-HT TRANSPORTER IN DEPRESSION

The serotonin transporter (5-HTT) is the pre-synaptic membrane receptor responsible for the re-uptake of serotonin from the synaptic cleft following neuro-transmission, in serotonergic neurones.

The transporter is therefore the target of the serotonergic anti-depressants including selective serotonin re-uptake inhibitors and the tri-cyclic anti-depressant, clomipramine (Anderson et al, 1992). It is also found in blood cells and the transporter proteins expressed in both brain and platelets have been shown to be identical (Lesch et al, 1993b).

1.7 GENETIC STUDIES IN PSYCHIATRY

1.7.1 Introduction

As discussed in the general introduction above, the family, adoption and twin studies have established a significant genetic contribution to the aetiology of the affective disorders although the specific chromosomes and genes involved are yet to be elucidated. It is now more apparent, however, that aetiology is multi-factorial and that both genetic and environmental factors are relevant (Kendler and Karkowski-Schuman, 1997). It is also evident that the genetic contribution is likely to be complex as single gene defects have not been consistently correlated in patient groups, in comparison with controls. It is therefore more likely that the genetic contribution relates to more than one system in a polygenetic model. Single genes and the polymorphic variation of genes with a recognized role in neuro-transmitter and other systems related to mood have been studied, both by rates within groups and by comparison between groups with either demographic (eg: gender or ethnicity effects) or treatment (eg: response versus non-response) differences.

1.7.2 Polymorphic variation

Polymorphic variation refers to the existence of 2 or more alleles of genes occurring at significant levels in the population (Strachan and Read, 1996). Interest in allelic variation exists in view of the potential for not only structural but also functional differences to occur. In the case of psychiatric disorders it is hoped that the understanding of either influences on the development of the condition or variations in response to treatment might be improved by the elucidation of any functional aspects of allelic variation.

Should the polymorphism in question not seem to relate directly to any known aspect of function it is then hoped that the variation might represent a marker with a possible linkage to a gene of actual effect and this would then be established by methods of linkage analysis (Owen, 1997).

In the following section and in three of the project chapters attention is focussed on the 5-HT_{2C} receptor and serotonin transporter from a genetic perspective, in view of the above mentioned links of the 5-HT_{2C} receptor with satiety and the reported correlation of one 5-HT_{2C} receptor polymorphism with response of schizophrenic patients to clozapine (See below). The 5-HTT has also been a focus in view of its pivotal role in the re-uptake of 5-HT from the synaptic cleft and the effect of anti-depressants on its function.

1.7.2.1 5-HT_{2C} RECEPTOR

Polymorphic variation

The human 5-HT_{2C} receptor has been localised to the long arm (q) of the X chromosome (Xq24). Complementary DNA (c-DNA) sequencing, achieved using reverse transcriptase, has revealed a C - G polymorphism (the cytosine base exchanged for guanine) at nucleotide 68 such that the amino acid serine is substituted for cysteine in the receptor protein at codon 23. The position of this polymorphism can be seen diagrammatically in Figure 1.4. (See below).

The proportion of the normal population who have the substitution is approximately 13 per cent in one study (Lappalainen et al, 1995), while the number of homozygous females is approximately one per cent. However, although this polymorphism has been studied extensively, there are now other described polymorphic variations including 2831 T/G and promoter variants.

Further work is now also continuing with the study of RNA-editing which occurs following transcription but prior to translation of m-RNA into proteins, leading to further modification of the final receptor protein structure (Fitzgerald, 1999).

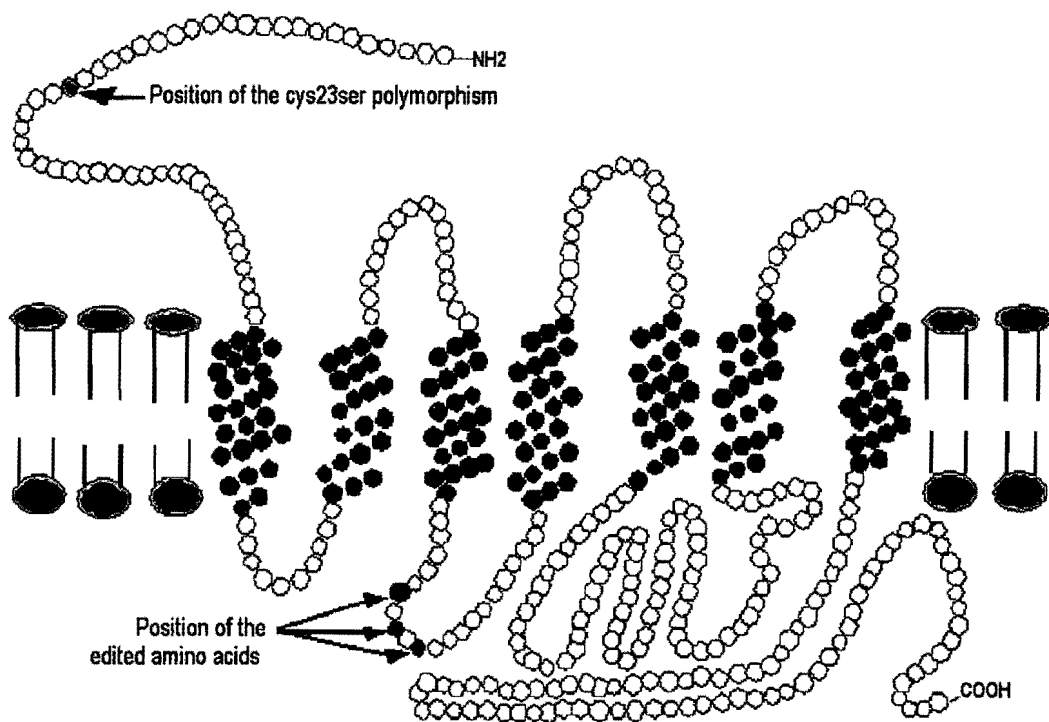


Figure 1.4: Diagrammatic representation of the 5-HT_{2C} receptor illustrating the seven transmembrane domain structure and the position of the cys23ser polymorphism. Editing of m-RNA (additional points shown) is discussed in the final chapter. Used, with permission, from Dr.M.Sodhi, PhD thesis (University of London).

Polymorphic variation has also been demonstrated for the 5-HT_{2A} receptor gene which is found on chromosome 13 (13q 14-21), with six reported polymorphisms including one causing a T - C substitution at codon 102. This substitution is silent, with conservation of the final receptor amino-acid sequence and is present in 40 per cent of the general population. Other 5-HT_{2A} polymorphisms include 452 His/Tyr as well as 1438 G/A.

Function

It is of clinical relevance to know whether two receptor variants are functionally different.

Although initial *in vitro* work on receptors expressed on xenopus oocytes showed equal concentration/response curves to 5-HT application, later work has shown a difference in binding affinity (Goldman et al, 1995). In the treatment of refractory schizophrenia by clozapine (which includes among its many pharmacological properties that of 5-HT_{2C} receptor blockade), as mentioned above, one study has shown significantly increased clinical response rates in patients possessing at least one serine substituted allele (Sodhi et al, 1995) but this was not supported in more recent studies (Masellis et al, 1995; Segman et al, 1997; Malhotra et al, 1998). However, one further weakly positive study has been reported by Arranz and colleagues and a recent meta-analysis has supported serine substitution correlating with response at a statistical significance of $p=0.02$ (Sodhi, personal communication) in a serine dominant model.

This finding refers only to those of caucasian ethnic origin and other explanations than simple changes in the function of 5-HT_{2C} receptor function need to be considered. These include the possibility of the allelic variant being in linkage disequilibrium with another gene coding for a functionally relevant protein or the serine substituted group being a marker for longer hospitalisation which the group on clozapine treatment would represent. Further association studies have been carried out with psychiatric patients, with a slightly greater but non-significant frequency of serine alleles being found in patients with Bipolar disorder and with later onset depressives (Gutierrez et al, 1996) as well as correlating with the presence of hallucinations in patients suffering from Alzheimers Disease (Holmes, 1998). A further explanation is that the polymorphism may alter the way in which clozapine binds to the 5-HT_{2C} receptor.

1.7.2.2 5-HT transporter

Polymorphic variation

The identity of the human platelet and brain serotonin transporter gene was established following sequence analysis of a c-DNA and the location was revealed to be on chromosome 17 (17q11.1-q12) (Lesch et al, 1993a and b) (See Figure 1.5 below). Subsequent molecular studies have revealed that the transporter gene has a number of polymorphic variants.

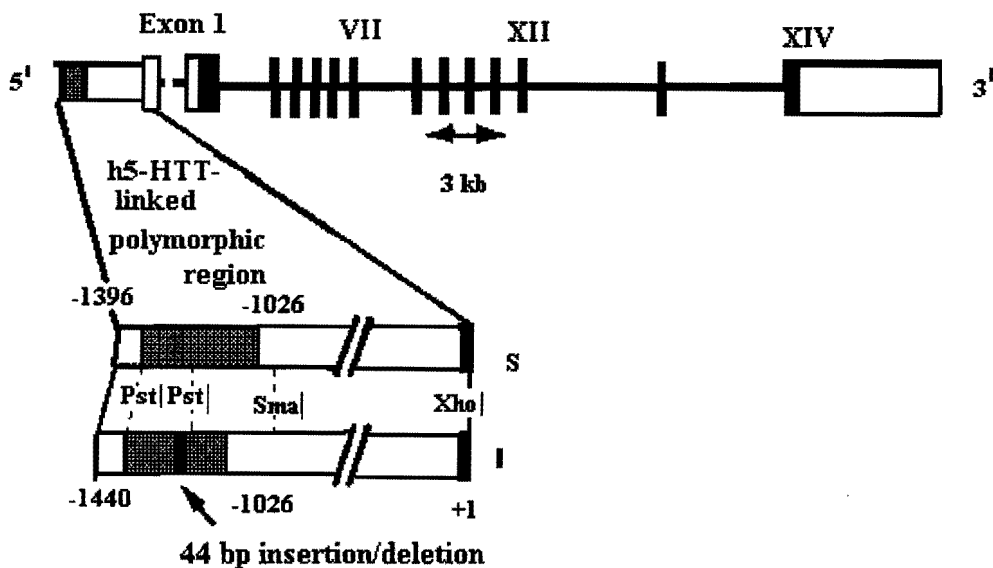


Figure 1.5: Diagrammatic representation of the human serotonin transporter gene (h-5HTT) illustrating the 44 base pair (bp) insertion/deletion in the promoter region. Reproduced with permission from Dr.K.P.Lesch.

One of these, in the promoter region for the transcriptional control of SLC6A4 (5-HTTLPR), consists of varying lengths of a repeat sequence of 20-23 bp long elements. It is a polymorphism with a 44-base-pair insertion/deletion, giving rise to a bi-allelic variant, with 16 repeats- 'long', and 14 repeats-'short' (l and s, respectively).

It causes a variation in the extent of tissue expression of the transporter (Heils et al, 1995) with the 'l' form being associated with increased rates of transporter site expression (Lesch et al 1996; Little et al, 1998) and the short, 's' version with lower rates of expression and activity, whether homozygous or heterozygous, implying that the short version is dominant.

The situation is more complicated than originally thought as it has been recently established that even the known 5-HTTLPR alleles have other subdivisions and further variants have been discovered, most recently ten novel alleles (Nakamura et al, 2000), leading to a recommendation that each of these alleles should also be considered in studies for a potential contribution to functional differences. Comparison of different mammalian groups has confirmed that the 5-HTTLPR is unique to humans and simian primates and it is estimated that a progenitor sequence may have been introduced into the genome approximately 40m years ago. Great apes such as the orangutan, gorilla and chimpanzee have an over-representation of 18 and 20 repeat elements (Lesch et al, 1998). In contrast, the majority of alleles seen in humans are either 14 or 16 repeat elements, while the above two are rare.

Further variability occurs in the comparison of different ethnic groups. Caucasians in one study were seen to have 32% 'l/l', 49% 'l/s' and 19% 's/s' (Lesch et al, 1996). In addition to the above polymorphism, a second polymorphism is found in intron 2 of the 5-HTT gene, a VNTR with 9, 10 or 12 repeats (Lesch et al, 1994).

Function

In establishing whether 5-HTTLPR alleles differ in their functional capacity, the finding referred to above involved the fusion of the 5-HTT gene promoter with a luciferase reporter gene and transfection into human lymphoblastoid cell lines (Lesch et al, 1996). In addition to the main finding - increased tissue transporter expression of the long ('l') allele, it was also established that 'l' homozygosity leads to higher concentrations of 5-HTT m-RNA and antagonist binding density.

In an analogous study in platelets, the 'l' variant was associated with more rapid initial platelet 5-HT uptake. However, the genotype did not affect platelet (³H)-paroxetine binding, affinity for (³H)5-HT or (³H)-paroxetine, or 5-HT content (Greenberg et al, 1999). In a post mortem study a genotype effect was also seen in dorsal raphe (¹²⁵I)CIT binding, with higher levels of presumed 5-HTT bound in ethanol abusers either homozygous for the short 's' allele or heterozygotes (Little et al, 1998).

A alternative means of investigating possible genotype effects has been through the measurement of metabolic break-down products of the major neuro-transmitters in the CSF. This method, widely used in the early investigation of the mono-amine hypotheses in psychopharmacology, has also been applied in the evaluation of possible transporter variations. Both of the two serotonin transporter polymorphisms discussed above were investigated by CSF analysis following the lumbar puncture of healthy volunteers.

For both the intronic and promoter polymorphisms significant relationships were found with MHPG levels but not with HVA and HIAA concentrations. As multiple comparisons were made, however, following a Bonferroni correction only the intronic difference remained significant (Jonsson et al, 1997). Problems exist with this finding, as 5-HT is usually metabolised to 5-HIAA and the proportion converted to MHPG is minimal, by comparison. Furthermore the intronic polymorphism is not yet known to have a functional significance.

Studies in psychiatric conditions

Affective Disorders

In one population based case-control study there was an association of the 5-HTTLPR short allele with affective (predominantly bipolar) illness (Collier et al, 1996a), but this was not replicated in three subsequent studies (Ohara et al, 1998; Bellivier et al, 1997; Rees et al, 1997). If depressive spectrum disorders are included, one family-based study of alcohol dependence showed preferential transmission of the short allele (Lichtermann et al, 2000). In a post-mortem sample there was a positive association of the long allele in depressed suicide victims (Du et al, 1999). In a symptom rating based study no genotype interaction with depressive symptomatology was found (Serretti et al, 1999) however, in another study, 'l' homozygotes showed an enhanced mood improvement after sleep deprivation (Benedetti et al, 1999). As regards the VNTR in intron 2, an initial study in Scotland reported an association between the 9-repeat allele and unipolar depression (Ogilvie et al, 1996) in the patient group compared with controls.

Other studies have failed to replicate this finding (Collier et al, 1996b; Stober et al, 1996; Bellivier et al, 1997). Two further studies were negative for the nine repeat allele (Rees et al, 1997; Kunugi et al, 1997) but found a positive association between the 12 repeat version and bipolar disorder, as first reported by Collier (1996b). This association was not reported by Ogilvie et al (1996), or in the three studies negative for the nine repeat allele cited above. Following from the above it can be seen that three studies have been positive for the 12 repeat allele association with affective disorder and four have been negative. In a chi-squared analysis of the combined replication findings, but excluding the one Japanese study for reasons relating to ethnic variability of population allele rates, a positive association was reported, but only when the Collier findings were included (Greenberg, 1998). $X^2 = 13.15$; $P < 0.01$. In one study of puerperal affective psychosis, an excess of the Stin2.12 variant was associated with a four-fold increased risk of psychosis (Coyle, 2000).

Further studies have focussed on a possible association with clinical response to antidepressant medication, as has been done in chapter four of this thesis. Work has focussed on two polymorphisms which occur in the serotonin transporter (5-HTT) gene – one in the promoter region (5-HTTLPR) and the other in intron 2 (Stin2). Some studies have detected allelic association between this gene and clinical response to SSRIs. In one study patients with the long polymorphic variant - 'l/l' and heterozygotes with the short and long - 's/l' - versions of the promoter region polymorphism were more likely to show an improved response to fluvoxamine, an effect abolished in the group also treated with pindolol (Smeraldi et al, 1998).

In two similar studies utilising paroxetine, an increased response rate was seen in subjects with at least one copy of the long variant of the 5-HTTLPR polymorphism in a study involving young adults (Zanardi et al, 2000) and in a further study involving an older group of patients. In the latter study a more rapid response was seen for those patients homozygous for 'l/l' promoter alleles compared to those with an 's' allele (heterozygotes or homozygotes) (Pollock et al , 2000). However, in a further study an enhanced clinical response has been reported in patients homozygous for the short version - 's/s' - of the 5-HTTLPR polymorphism, and in homozygotes with the Stin2.12 version of the intron 2 polymorphism (Kim et al, 2000).

Neuroticism/anxiety disorders

Another approach to elucidating the relevance of polymorphic variation to psychiatric syndromes involves the use of large populations to carry out association studies between polymorphic variation and personality traits such as harm avoidance or neuroticism. An increase in neuroticism scores was seen to a greater extent in individuals with the short arm of the 5-HTTLPR polymorphism in one large study (Lesch et al, 1996), however in two smaller studies no association was seen with the short allele in either neuroticism or harm avoidance (Ball et al, 1997; Ebstein et al, 1997).

In other psychiatric populations studies of association have also been negative. Two studies involving patients with panic disorder showed no association with this 5-HTTLPR genotype (Deckert et al, 1997; Matsushita et al, 1997).

Other studies of anxiety related factors include a Japanese sample with a trend towards an association (Nakamura et al, 1997), and a Finnish study with equivocal findings (Mazzanti et al, 1998), however a study from Israel showed an interaction with the D4DR Dopamine polymorphism (Ebstein et al, 1998). Two further studies showed positive associations (Ricketts et al, 1998; Osher et al, 2000).

Obsessive Compulsive Disorder

Although both the central importance of serotonergic drugs in the treatment of OCD and a significant genetic contribution as inferred from the twin and family studies would suggest that polymorphic variation of the serotonin transporter gene might be relevant, two studies have not supported this (Altemus et al, 1996; Di Bella, 1996). In one 5-HTTLPR study a trend was seen towards patients being homozygous more frequently than controls and if controls without formal diagnostic interviews were excluded the finding was significant (Billett et al, 1997). This was replicated in two further studies where population-based analysis showed an excess of l/l alleles in the OCD group (Bengel et al, 1999; McDougle et al; 1998).

Autistic syndromes

Preliminary evidence of linkage and association between the 5-HTT gene and autism was reported from a study including 86 trios (parents and probands) where preferential transmission of the short variant was found and, in spite of the lack of any linkage between the condition and the intron 2 polymorphism, a multi-alleleic TDT confirmed the significance of the region (Cook et al, 1997).

Unfortunately, an attempted replication by a different group showed the preferential transmission of the long 5-HTTLPR variant (Klauck et al, 1997), thereby failing to clarify the issue. One study analysed rates in schizophrenic patients (Stober et al, 1998), but neither the 5-HTTLPR polymorphism or the intron 2 polymorphism genotypes or allele frequencies varied between the schizophrenia group and control group.

While the above chapter has covered the background knowledge relating to the field of inquiry and has argued the case for a likely involvement of serotonin receptors in the drug treatment of depression and psychosis, the following chapter summarises the methodology used to test the hypotheses derived from the theoretical model.

CHAPTER 2

EXPERIMENTAL APPROACH AND GENERAL METHODS

2.1 NEURO-ENDOCRINE CHALLENGE TESTS

2.1.1 Subject selection

In the three projects involving subjects rather than psychiatric patients (paroxetine study, 5-HT_{2C} function study, and clomipramine study) volunteers were included following an interview regarding clinical details. Specific inclusion criteria included: age between 18 and 50 and absence of personal history of psychiatric illness according to diagnostic criteria. Exclusions were: current use of illicit substances or excessive alcohol intake; particular sensitivities to the nature of the endocrine challenge procedure e.g. needle insertion or nausea immediately following cannulation and any significant organic condition. In the m-CPP challenge tests subjects with a history of migraine were excluded. In the patient study (Ch.4) individuals were questioned regarding different aspects of possible psychiatric symptoms as delineated in the schedule for clinical interviews (SCID) in order to derive a diagnosis according to the Diagnostic and Statistical Manual – 3rd edition, revised or 4th edition (DSM3R or IV).

2.1.2 Randomisation

In all three studies involving healthy subjects the sequence of neuro-endocrine test challenges was dictated by randomisation and included a balanced order design. In the 5-HT_{2C} function study both the subjects and investigators were blind to the day of active drug administration vs placebo day however in the paroxetine and clomipramine studies, in view of the intravenous administration, blinding only occurred from the subjects' point of view (single-blinded) as the investigators were aware of the content of the intravenous syringe. The randomisation procedure involved a colleague in the Psychopharmacology Unit drawing up a code using randomisation tables as provided by a member of the Oxford University Department of Mathematics. The codes were kept under lock and key and only subsequently consulted when the subjects had been tested. Code breaking and analysis of results occurred jointly between at least two members of the department involved with each specific study.

2.1.3 Timing of tests

It was thought that a minimum gap of one day between the tests would obviate any possible lasting drug effects as all of the half-lives of the drugs involved are less than 16 hours. It has not previously been established in related experiments that there are any residual effects on the hormonal and behavioural responses the following day, leading to an order effect.

2.1.3.1 Gender issues

As m-CPP challenges have been found to have differing behavioural and hormonal effects according to the menstrual cycle (Cowen et al, 1995), female subjects were only tested during the follicular phase (days 1-14) of the menstrual cycle where 'day one' is the first day of menses. Days were, however, calculated to ensure that both test days (placebo and drug) could be completed in the early part of the follicular phase.

2.1.3.2 Effect on receptors

It is unlikely that any receptor effects could occur following the administration of an active substance on the first test day that would have significantly altered responses on the second test day if the order determined by randomisation stipulated that the placebo day should follow the active substance day. Serotonin receptor changes have been described following the administration of anti-depressants and with chronic m-CPP use but these effects characteristically occur after weeks, rather than days taking the drugs. Further studies of relevance to this question include receptor studies where drug effects appear to occur via a greater number of mechanisms than post-synaptic neuro-transmitter effects alone. Intra-cellular responses also occur, leading to changes in gene expression and subsequent influences on trophic and neuro-adaptive changes. In one study four days of imipramine treatment of rats lead to a significant increase of 5-HT_{2C} m-RNA levels but a single dose only caused a slight increase (Tohda and Watanabe, 1996).

Although it is assumed that periods of time longer than the lag between the test days in these studies will be needed to see significant and lasting effects on receptors, the specific nature of the changes and time periods involved are still under investigation (Barnes and Sharp, 1999).

2.1.3.3 Separation of test days

The 5-HT_{2C} function tests occurred with a minimum gap between each pair of test days of two days, as did the clomipramine study. In the paroxetine study a separation of 1 day was permitted and the paroxetine was stopped following the second test day, after the three week period of paroxetine treatment.

2.1.4 Setting of tests

2.1.4.1 Cannulation procedure

Subjects attended the Psychopharmacology Research Unit at Littlemore Hospital or the Warneford Hospital, Oxford, after an overnight fast and were cannulated half an hour before the first samples were withdrawn (Venflon cannulae, 16 - 18 gauge). After this rest period samples were withdrawn to analyse baseline hormonal levels. Following this heparinised saline was used to maintain patency of the venflon in conjunction with a butterfly set in order to facilitate multiple sampling over time.

Heparin was used in a mixture with normal saline (10mls normal saline to 0.02mg of heparin). The heparin was stored in a refrigerator prior to testing and the mixed heparinised saline was discarded following the completion of each test. During sampling, an initial blood specimen of 10ml was withdrawn to be discarded, following which the sample for analysis was withdrawn.

2.1.4.2 Pre-test protocol

Patients and subjects were transported to the Psychopharmacology Unit, by taxi-cab as some subjects experience anxiety or somnolence following the test and this might impair concentration for driving. It was also hoped that being brought to the Research Unit might be less stressful than driving and therefore contribute to a more rapid stabilisation of the hormonal stress response.

As elevations in the PRL level have been found following the stress of cannulation it was necessary to leave a minimum half hour period of adjustment following cannulation and samples were therefore withdrawn at both '-30' minutes as well as at time '0' (base-line), in order to assess possible outliers exhibiting major stress effects on the hormonal levels.

2.1.5 Sample and specimen storage

2.1.5.1 Centrifugation

Lithium heparin tubes were used for blood sample storage following extraction from the intravenous cannula. 20mls of blood was extracted at each time point and the samples were stored in ice at the bedside until the study was completed. Plasma was separated by centrifugation at 2000RPM for 10 minutes.

2.1.5.2 Refrigeration

Following centrifugation the super-natant was removed by pipette and placed in further sample tubes without lithium. These plastic tubes were then frozen and stored at -22°C until assay. The samples were stored in wire mesh containers in the freezers until required for analysis of the contents. Plastic containers do not perish or break which ensures the maximum number of samples are available for study. The sample containers are labelled according to test and the tubes by code to ensure confidentiality for the subjects and blindness in relation to laboratory staff involved in multiple projects.

2.2 SUBJECT ASSESSMENTS

2.2.1 Rating scales in depression

In the treatment response study involving depressed patients two scales were utilised. The Beck Depression Inventory (BDI) is a self-rated scale composed of 21 categories of attitudes and statements, each category consisting of 4-5 self-evaluative statements (See Appendix 1). Numerical values between 0 and 3 are assigned to each category statement in order to denote the measure of severity, 3 being the most severe. Scores of 11 or less suggest no depression is present; scores between 11 and 18 suggest mild depression; 19 to 29 suggests moderate depression and 30 and over is severe depression. The Hamilton Depression Scale (HAM-D) provides a method of scoring the severity of symptoms and is completed by a clinical interviewer who has been trained in the eliciting of psychopathological features but is not necessarily a psychiatrist. Symptoms are rated according to a series of scores ranging from 2 to 4 as a maximum for each of 17 items (See Appendix 2). The following categories apply:

Total score	Category
0-7	No depression
8-12	Mild depression
13-17	Mild to moderate
18-29	Moderate depression
30-52	Moderate to severe depression

Investigators testing response to anti-depressant treatments usually take a level of 8 on the BDI (Beck et al, 1961) and 7 on the HAM-D (Hamilton, 1960) as a measure of resolution. A further scale used by collaborators in the SSRI response study (See Chapter

4) is the Montgomery and Asberg Depression Rating Scale (MADRS) (Montgomery, 1979) which has been shown to have greater sensitivity to change over time. As with the other scales, response can be measured according to a reduction by a specified percentage of the original score or a set number of points.

2.2.2 Visual Analogue Scales

Scales of this type have been used extensively in neuro-endocrine testing paradigms and are used to record physiological and behavioural responses to the particular probe in question (Silverstone et al, 1994; Cowen et al, 1995). Physiological measures include sweatiness, light-headedness and nausea whereas psychological measures include mood states such as “happy” or “sad”. For the purposes of the 5-HT_{2C} function test appetite was also rated on both days. The rating scales consisted of 10cm lines divided into 10 equal sections by a scale from 0-100 where 0 represents “not at all” and 100 represents “very much”. (See Appendix 3)

The visual analogue scales are administered by first showing the subject the card. The subject then notes the point on the card which corresponds to the physiological or psychological measure. This point is recorded on a separate sheet and not directly on the visual analogue scale. In addition to the visual analogue scales mentioned, observation was made of any untoward physiological response to the challenge e.g. subjects feeling faint or vomiting, although in practice this did not occur.

2.2.3 Routine observations

Throughout the neuro-endocrine challenge tests measurements were taken of pulse and blood pressure by automatic recording (Dynamap, model 3341A, Sage instruments) on an hourly basis. These measurements were also routinely taken before the first sample of blood was extracted, as a baseline measure. Temperature readings were taken as part of the experiments but were not an essential part of the monitoring of physical signs from a health perspective. Subjects had all been nil per mouth from midnight on the preceding night however sips of water were permitted.

2.3 ENDOCRINE ASSAYS

2.3.1 Prolactin assay

Determination of prolactin levels in human plasma was carried out by solid phase, two site, immunoradiometric assay.

2.3.1.1 Materials

Prolactin reference material available in horse serum was purchased from SkyBio (Wyboston, Berkshire UK). The monoclonal radiolabelled prolactin antibody I¹²⁵ (raised from the mouse) and the anti-prolactin solid-phase (raised in the sheep) were also purchased from SkyBio. 'Solid-phase' refers to the second anti-body or anti-prolactin which binds to the antigen epitope (sub-component of an antigen).

All solvents and chemical reagents used were of the highest grade available. The stock phosphate buffer consisted of 0.1M (15.3g) sodium dihydrogen orthophosphate monohydrate, 0.3M (57.1g) of disodium hydrogen orthophosphate dihydrate and 2mls of 1% thiomersol (a reagent which acts as an antibacterial agent, prolonging the stability of the stock solution), dissolved in 1 litre of high purity water. The pH of the solution was 7.4.

The wash buffer consisted of 100mls of stock phosphate buffer and 5mls of Tween 20 (Merk Eurolab Ltd. UK), which is a surfactant added to the wash buffer to prevent other constituents from plasma and solution matrices from non-specifically binding to the solid-phase and labelled antibody binding sites.

The assay buffer consisted of 1% bovine serum albumin (BSA) (Sigma-Aldrich, UK) in 100mls of wash buffer. This solution prevents endogenous plasma matrix and other solution constituents, including fractions of radiolabelled antibody, from binding to the plastic surfaces of test tubes. This was to ensure the removal of erroneous radioactivity counts in the precipitate (see below) and the precise identification of the bound tracer-antibody complex, thus keeping the NSB to a minimum.

2.3.1.2 Methods

All samples, standards and quality controls were analysed in duplicate over a two day period.

The standard curve range used for this assay included the concentrations of 0, 40, 80, 160, 400, 800, 1600 and 4000 milli-international units per litre (mIU/L). The prolactin serum standards were diluted in 1ml of high purity water before use according to the supplier's specifications.

Day 1

1. 50µl of plasma sample, standards, totals (see below) and quality controls were added to clearly labelled 75 x 10mm polypropylene test tubes (Sarstedt Ltd.UK).
2. 50µl of the solid-phase (anti-prolactin) and 400ul of assay buffer was added to all tubes except the totals.
3. Vortexing then incubation was carried out at room temperature on rotary mixers for 3-5hrs.
4. 2mls of wash buffer was added to all tubes and centrifuged for 5mins at 2100 RPM.
5. The supernatant was decanted to waste, leaving the prolactin bound solid-phase precipitate remaining at the bottom of the test tubes.
6. 50µl of the radiolabelled antibody (I^{125} -antibody) and 400µl of assay buffer was added.
7. Vortexing and incubation was again carried out, overnight on rotary mixers.

Day 2

1. 2mls of wash buffer was added and centrifuged for 5minutes at 2100 RPM.
2. The supernatant was decanted to waste and the washing step repeated.
3. Finally, the prolactin bound radiolabelled-antibody complex was counted for 5mins on a RiaStar Packard gamma ray counter (Packard Bioscience. Berkshire UK).

The totals are those tubes that only contain radiolabelled antibody I¹²⁵ solution prepared in assay buffer. This was used as a measure of the total amount of radioactivity to be added to each test tube. The optimal level of radioactivity from the labelled antibody for this assay is approximately 60,000 counts per minute (cpm) as determined by a gamma counter.

Table 2.1: Inter-assay coefficients of variation (% CV) for the determination of prolactin in human plasma (n = 20).

	Mean	Standard deviation	CV (%)
Low	114.1	9.9	8.7
High	361.9	15.8	4.4

Table 2.2: Intra-assay coefficients of variation (CV) for the determination of prolactin in human plasma (n = 10).

	Mean	Standard deviation	CV (%)
Low	71.3	6.4	9.0
High	825.1	43.5	5.3

The statistics for both the inter-assay variation data were calculated from 20 sets of replicate samples for each low and high QC's. For the intra-assay data 10 sets were used (See tables 2.1 and 2.2 above).

2.3.2 Cortisol assay

Determination of hydrocortisone (cortisol) in these studies was performed using radioimmuno-assay according to the following protocol.

2.3.2.1 Materials

Hydrocortisone reference material was purchased from Sigma (Sigma-Aldrich, UK) and the radiolabelled cortisol (^{125}I cortisol) stock solution was obtained from Amersham Biosciences (Bucks, UK Ltd.).

The assay buffer consisted of 8.4g of citric acid monohydrate, 8.5g of disodium hydrogen orthophosphate dihydrate and 1g of gelatin dissolved in 1 litre of high purity water. The pH of the solution was 4.0.

The cortisol antibody raised in the rabbit was purchased from BioClin (Bioclinical Services, Cardiff, UK). A second antibody, "SAC-CEL", is another antibody which attaches itself to the bound radiolabelled cortisol antibody complex raised in the rabbit. This was used to separate and immobilize by precipitation, the bound antibody complex from the free "non-bound" radiolabelled cortisol and was purchased from IDS (Baldon Ltd., Tyne & Wear, UK).

2.3.2.2 Methods

The cortisol antibody was diluted in 1ml of high purity water, then divided into 200 μ l aliquots and stored at -20°C . When required for assay, a 200 μ l solution of the diluted antibody was retrieved and further diluted with 19.5mls of the assay buffer prior to use.

All samples, standards and quality controls were analysed in duplicate over a two day period. The standard curve range used for this assay includes the concentrations of 0, 0.1, 0.5, 1, 5, 25 and 75 micrograms per 100 millilitres ($\mu\text{g}/\text{cl}$).

Day 1

1. 25 μ l of plasma sample, standards and quality controls.
2. 75 μ l and 25 μ l of assay buffer in tubes were labelled NSB and “zero” respectively.
3. 50 μ l of cortisol antibody was added to all tubes except those labelled NSB and totals (see below).
4. All tubes were incubated at room temperature for 90mins.
5. 200 μ l of the of the diluted I^{125} cortisol solution was added to all tubes.

Day 2

1. 100 μ l of the solid-phase (SAC-CEL) was added to all tubes except the ‘totals’ and incubated for 30 mins at room temperature.
2. 1ml of high purity water was added to all tubes except the ‘totals’ and centrifuged at 2500RPM for 15mins at 4°C .

3. Supernatant containing all of the “non-bound” I^{125} cortisol was decanted to waste, leaving the bound I^{125} cortisol antibody complex to be counted for 5 minutes on the gamma counter.

The ‘totals’ are those tubes that only contain radiolabelled I^{125} solution prepared in assay buffer. This was used as a measure of the total amount of radioactivity to be added to each test tube. The I^{125} cortisol solution was diluted in assay buffer to achieve the optimal level of radioactivity (approximately 15,000 cpm) required for this assay.

Table 2.3: Inter-assay coefficients of variation (CV) for the determination of cortisol in human plasma (n = 20).

	Mean	Standard deviation	CV (%)
Low	9.9	1.0	7.7
High	31.8	4.6	11.3

Table 2.4: Intra-assay coefficients of variation (CV) for the determination of cortisol in human plasma (n = 10).

	Mean	Standard deviation	CV (%)
Low	3.35	0.19	5.6
High	21.5	1.9	8.7

The statistics for the inter-assay variation data were calculated from 20 sets of replicate samples for both low and high QC’s. The intra-assay data set was calculated from 10 sets of samples (See tables 2.3 and 2.4 above).

2.4 PHARMACOLOGICAL ASSAYS

2.4.1 m-CPP and paroxetine measurement

The m-CPP and paroxetine levels were determined by reverse phase High Performance Liquid Chromatography (HPLC) (Franklin, 1992). The end-point detection was achieved by utilising coulometric electrochemical detection (ESA, Bedford , MA, USA). The operating potentials selected for detectors 1 & 2 were 750 and 930 millivolts respectively. Detector 1 was used to preoxidise any extractable compounds that are of no interest and might interfere with the analytes at detector 2, the analytical electrode. The analytical column used was a “mixed mode” (ODS CN) (a combination of a C18 and cyanonitrile material) (Capital HPLC, Edinburgh, UK). The mobile phase consisted of a 74 % methanol in phosphate buffer (v/v) with the flow rate set at 1.00ml/min.

The isolation of both compounds from the plasma matrix procedure involved utilising solid-phase extraction (SPE) technologies. To both samples and standards an internal standard drug was added to monitor both the extraction recovery and the detector response variation. Plasma samples were then loaded onto carboxymethyl (CBA) solid phase SPE cartridges after conditioning with a phosphate buffer and the analytes were eluted with an ammonia / methanol solvent into glass collection vials. The solvent was evaporated to dryness and reconstituted in the mobile phase.

An aliquot of this solution was injected onto the HPLC analytical column for chromatographic separation and quantitation.

Standard curves were freshly prepared daily and consisted of concentration points over the range of 0 – 50ng/ml. The linearity of both the extraction procedure and the detector response was verified over the range of the standard curves. This was determined by measuring pooled drug-free plasma spiked with known amounts of m-CPP, paroxetine and the internal standard drug. The calibration curves for both compounds were calculated by peak-height ratio's relative to the internal standard drug.

The concentrations of both compounds were assessed by interpolation of the standard curves using the general linear equation $y = ax + b$. The mean linear equations for the calibration curves for m-CPP were $y = 0.035x + 0.090$ ($r = 0.994$; $n = 5$) and for paroxetine were $y = 0.024x + 0.078$ ($r = 0.995$; $n = 4$).

The mean intra-assay CV was 4.7% for m-CPP ($n = 15$) and 5.6% for paroxetine ($n = 15$) for a spiked plasma quality control containing 10ng/ml for both compounds. The mean inter-assay CV was 7% ($n = 10$). The lowest level of quantitation (LLQ) for both compounds was 1ng/ml.

2.4.2 Clomipramine Assay

The clomipramine and its major metabolite nor-clomipramine levels were determined by reverse phase HPLC utilising ultraviolet-visible (UV/VIS) (Jasco, UK) end point detection (Odontiadis, 1996). The detector wavelength was set at 215 nanometres (nm). The analytical column used was a cyano-nitrile (CN) (Capital HPLC, Edinburgh, UK) and the mobile phase used consisted of 40% acetonitrile in a phosphate buffer (v/v) with the flow rate set at 1.5mls /min.

The isolation of both compounds from the plasma matrix procedure involved utilising SPE technologies. To both samples and standards an internal standard drug was added to monitor both the extraction recovery and the detector response variation. Plasma samples were then loaded onto carboxymethyl (CBA) solid phase SPE cartridges after conditioning with a phosphate buffer and the analytes were eluted with an ammonia / methanol solvent into glass collection vials. The solvent was evaporated to dryness and reconstituted in an acetonitrile / distilled water solution. An aliquot of this solution was injected onto the HPLC analytical column for chromatographic separation and quantitation.

Standard curves were freshly prepared on a daily basis and consisted of concentration points over the range of 0 – 2000ng/ml. The linearity of both the extraction procedure and the detector response was verified over the range of the standard curves.

This was determined by measuring pooled drug-free plasma spiked with known amounts of clomipramine, nor-clomipramine and the internal standard drug. The calibration curves for both compounds were calculated by peak-height ratio relative to the internal standard drug. The concentrations of both compounds were assessed by interpolation of the standard curves using the general linear equation $y = ax + b$. The mean linear equations for the calibration curves for clomipramine were $y = 0.00245x + 0.031$ ($r = 0.998$; $n = 3$) and for nor-clomipramine $y = 0.00164x + 0.020$ ($r = 0.999$; $n = 3$). The lowest level of quantitation (LLQ) for both compounds was 10ng/ml.

2.5 GENETIC ANALYSIS

Analysis of samples to establish details of their genetic polymorphic variation was necessary in the SSRI response study, the 5-HT_{2C} function study and the clomipramine study but not the paroxetine study.

2.5.1 DNA extraction

Blood samples or buccal smears from patients and subjects were refrigerated initially in plastic tubes at -70 °C in order to avoid deterioration of DNA. Genomic DNA was subsequently isolated from lymphocytes or from buccal epidermal cells using commercial kits (Nucleon) in batches, and stored at -20 °C prior to use.

2.5.2 5-HT_{2C} polymorphism determination

Polymerase chain reaction (PCR) is a rapid, in vitro technique by which selective amplification of a target DNA sequence within a more heterogenous group of DNA sequences can be achieved. In order for this to occur, known sequence information from the DNA target is used to generate two primer sequences, called amplimers, which bind to the c-DNA regions immediately adjacent to the target region. A heat stable DNA polymerase is required and for these experiments the Taq polymerase, obtained from the hot-spring micro-organism *Thermus aquaticus* is used (Strachan and Read, 1996).

Extracts were subjected to PCR with oligodeoxynucleotide primers corresponding to nucleotide positions 101 to 121 (5'-CACCTAATTGGCCTATTGGTT-3') and 406 to 386 (5'-AAGGATTGCCAGGAGAGACAG-3') of the human 5-HT_{2C} receptor gene (Stan et al, 1994). Reactions were performed in amplification buffer (Perkin Elmer) containing 20-100 ng DNA, 250µM dNTPs, 400nM of each primer and 1 unit of Amplitaq (Perkin Elmer), in a final volume of 20µl. Amplification consisted of 35 cycles of 94°C for 45 seconds, 55 °C for 1 min and 72 °C for 1.5 min. The genotype of the 305bp PCR products was initially determined by a 'dot-blot' method. Amplimers were denatured at 94°C for 5min and quenched on ice for a further 5 min. Samples were pipetted onto nylon membrane filters, dried at room temperature and cross-linked by baking the membranes at 80°C for 90 min. The filters were then hybridized over-night with [32P]-dATP labelled oligonucleotide probes specific for the cysteine or serine variant.

They were then washed in stringent conditions according to standard protocols. Blots were apposed against X-ray film for 24-48 hours so that allele specific signals could be visualized. The genotypes of each subject were confirmed using standard restriction fragment length polymorphism (RFLP) methods (Segman et al, 1997; Lappalainen et al, 1995).

2.5.3 Serotonin Transporter polymorphism analysis

The amplification of the genomic DNA isolated as described above (see section 2.5.2) was performed with oligonucleotides corresponding to nucleotide positions -1416 to -1397 (5'-GGCGTTGCCGCTCTGAATGC-3') and -910 to -888 (5'-GAGGGACTGAGCTGGACAACCAC-3') of the gene encoding the serotonin transporter transcriptional control region (5-HTTLPR) (Lesch et al, 1996; Klauck et al, 1997). Samples were added to 20 µl amplification buffer (Perkin Elmer) containing 20-100 ng DNA, 200µM dNTPs (dGTP/7-deaza-2-dGTP=1:1), 0.5µM primers, 1.5mM MgCl₂, 0.5 U Taq and 12% DMSO. Using the method previously described by Klauck and colleagues (1997), initial denaturation was at 95°C for 3 minutes followed by 2 cycles at 95°C (30s), 63°C (30s), and 72°C (1min), two cycles with annealing at 62°C (30s) and 35 cycles with annealing at 61°C (30s), with a final extension of 10 mins at 72°C. 10 µl of the reaction mix was electrophoresed through 3% agarose gels containing ethidium bromide. Markers of DNA molecular weights (Sigma) were electrophoresed adjacent to samples.

The long form ('l') of the 5-HTTPLR was identified as a 16 repeat product, and the short form ('s') as a 14 repeat product.

2.6 TEST MEAL PROCEDURE

2.6.1 Selection of food options

At the initial screening interview, once the subject had agreed to take part in the 5-HT_{2C} study, a food selection sheet was completed (See Appendix 4). This enabled the individual to choose sandwiches which they would normally find palatable i.e. a choice between brown and white bread, a choice between mayonnaise or no mayonnaise, cucumber or no cucumber, but margarine was standard. Optional fillings were tuna chunks, cheese, fine sliced chicken and ham.

The subjects were asked to choose a first and second choice of the filling. The second choice was used for a preliminary test meal at the time of the physical examination as part of the health screening procedure, during which time questionnaires were also completed. The subjects were alone in an interview room with a table and the sandwiches were prepared according to a standard protocol, in order to be iso-caloric (See next section).

2.6.2 Standardisation of preparation

The bread was weighed previously and specific amounts of margarine were used with a previously known mass. A set number of slices of ham and chicken were used as well as a set proportion of tuna, if chosen. Cheese was bought in grated form and weighed in order to be iso-caloric. Mayonnaise was measured in cooking scoops to ensure consistency of amount. The sandwiches were prepared immediately before consumption and presented to excess i.e. 5 rounds divided into 4 quarters. The sandwiches were provided with no garnishing and only a glass of water accompanied them. The sandwiches were provided with the instruction that there was no time limit and the individual must consume the quantity dictated by their appetite rather than by any personal notion of study aims or by reference to previous occasions.

2.6.3 Results of food consumed

Once the individual had completed the meal they notified members of staff. At this stage the remaining quarters were counted and the number of quarters consumed was determined. Quarters which had been started but not finished were counted as full quarters. At the time of the two neuro-endocrine challenge days the first choice of food was given to the subject unless practical considerations prevented this in which case the second choice was again administered. This was then repeated on the second day of testing.

Measurement of the quantity eaten was evaluated both by number of quarters consumed as well as by calorie content based on calculations concerning the specific food stuffs chosen. Although sandwiches were iso-calorific on both test days, for each person, the amount varied between individuals, depending on their specific choice of food.

2.7 PATIENT SELECTION

2.7.1 Inclusion and exclusion criteria

The Psychopharmacology Research Unit, previously based at Littlemore Hospital, Oxford has been recruiting patients suffering from mood disorders and obsessive compulsive disorder for a number of years. In order to facilitate recruitment for the studies clinical research workers have been linked with general practices in and around Oxford. These clinical researchers are all psychiatrists in higher training. They hold clinics in the surgeries and patients are referred by the general practitioners if it is thought that a psychiatric assessment and further management may be beneficial. As these studies have been carried out over a number of years there is a large database of patients suffering from major depression (DSM3R and DSMIV) who have been treated by psychiatrists attached to the unit and who have agreed to take part in research projects. As clinical information is available regarding the treatment of these individuals, the procurement of samples for genetic analysis allowed a correlation of clinical response with genetic variations.

Following approval of the study through the local Ethics Committee (OPREC) patients were contacted to see whether they would be prepared to take part in the SSRI response study (Chapter 4). Patients were contacted by telephone where possible and, failing this, by letter. Patients were then asked if they were prepared to attend the unit where a blood sample or buccal swab would be taken. Alternatively a research nurse or one of the psychiatrists involved in the project would ask the patients to attend the general practice surgery at the time of a subsequent research clinic.

Those patients who were prepared to participate but were unwilling to attend for a blood sample were sent a buccal swab pack by post and asked to return this in a prepaid first class envelope, following which the swab material was frozen. The above procedure enabled a retrospective collection of a number of patient samples for genetic analysis, following which the genotypes were linked with clinical information from the case notes as well as by interview for those able to attend.

A similar process was carried out by the research team at the Neuroscience Centre, Elizabeth II Hospital, Welwyn Garden City. They also had information regarding patients who had been treated on SSRIs with response determined by the MADRS rating scale (Montgomery, 1979). This team was able to recruit patients to participate and provide a number of samples for genetic analysis as well as clinical information regarding treatment response/non-response according to the CGI used in Oxford.

A prospective arm of the study involved the further recruitment of patients suffering from major depression, who provided blood samples at first assessment or following a period of treatment on antidepressant medication. This was carried out in the general practice surgeries by clinical researchers from the unit and the response was rated according to the CGI criteria.

Patients were also recruited during their attendance at the Warneford Hospital Mood Disorders Clinic as well as from outpatient clinics at the Warneford Hospital where psychiatrists with previous involvement with studies in the research unit were involved in the follow up of patients.

2.7.2 Screening interview

At the initial clinical interview routine information regarding demographic details, clinical history and current presentation were recorded. Patients were diagnosed according to the diagnostic criteria of DSM IV for major depressive episode, single episode or recurrent.

Patients were not asked to provide a blood sample if there was evidence of an organic condition or if alcohol or illicit substances appeared to play a major role in the patient's presentation and it was thought that comorbidity for other psychiatric or medical diagnoses might be present. If it was not possible to determine this at the initial interview a subsequent decision was made at further follow-up appointments.

2.7.3 Diagnostic procedures

Where patients were interviewed it was confirmed that they fulfilled diagnostic criteria for major depression. Where patients were not prepared to attend for a further interview the case notes were examined and a case note diagnosis of major depression was made according to DSM3R or DSMIV criteria. Both scales were used as the study commenced when DSM3R was in use but continued after DSMIV was introduced. This stage of diagnosis was necessary as psychiatrists attached to the practices had also seen patients who did not agree to take part in further research studies at the time but were in any case treated according to usual clinical protocols. A number of these patients subsequently agreed to provide samples for genetic analysis, but were not prepared to attend for further interview.

A small proportion of patients described co-morbid psychiatric disorders including anorexia or bulimia nervosa but these patients were not excluded unless the co-morbid diagnosis appeared to have more relevance to the overall clinical state than the major depressive disorder itself.

Patients included those with unipolar and bipolar depression but the diagnosis of a major depressive episode needed to be confirmed in both groups. History of a manic episode therefore did not exclude involvement per se as a number of patients with a history of bipolar disorder were found to respond to SSRI treatment clinically.

2.7.4 Informed consent

Information sheets were provided at the screening interview or subsequently if the patient remained in ongoing treatment. Following the provision of information, both verbally and by an information sheet, a signed consent sheet was obtained.

Patients who did not attend for interview but who had previously been involved in research studies in the unit were sent an information sheet with a buccal swab kit and those patients who had consented by telephone subsequently returned samples in the majority of cases. Those patients who were not prepared to attend the unit did not sign consent sheets and it was agreed by the Ethics Committee that furnishing the sample by post following the provision of information would be regarded as a consent procedure.

2.7.5 Physical examination

Those patients and subjects attending for neuro-endocrine research studies were examined to assess physical health prior to the research studies, however patients attending for routine clinical assessment without further involvement in studies other than the SSRI response study were not examined physically but rather, if there was any concern about the organic contributing factors in the case, a request was made to the general practitioner to follow up the physical condition of the patient.

2.8 DATA MANAGEMENT

2.8.1 Information collected

Patients who had previously been involved in research studies at the unit had rating scales completed at the time of the study including the Beck Inventory, the Ham-D depression scale and general clinical information. The Hertfordshire Neuroscience Group used the MADRS scores for the generation of CGI's relating to response and patients completed questionnaires regarding their personal psychiatric history as well as other questionnaires detailing family history, relating to composition as well as to family psychiatric history. Further demographic information was recorded on questionnaires however a history of psychiatric illness in the family was not regarded as a specific exclusion criterion for involvement in the studies.

2.8.2 Clinical Global Impression Scale

As the information relating to patient response to SSRIs derived from different forms of information, including rating scales that were not standardised across the entire group, all information regarding patients was processed to generate a clinical global impression (CGI) as widely used in clinical trials (Newhouse and Richter, 1994). Clinical information at initial interview, follow-up information, rating scales and personal knowledge of the patients themselves allowed the CGI to be completed. The CGI included four levels (See Table 2.5), the most frequently used set of divisions in depression treatment trials.

Table 2.5: Clinical Global Impression Scale (CGI) which combines information from different sources to generate a single score relating to clinical progress.

Score	Clinical response attained
1	Marked response
2	Good response
3	Poor response
4	No change or deterioration

Level one was marked or excellent response, level two good response, level three poor or minor response and level four - no change or deterioration. The period considered for response was 4-6 weeks on an appropriate dosage regime. In all cases it can therefore be seen that the clinicians involved were responsible for generating the CGI ratings. Where there were any concerns about whether a patient fulfilled criteria for inclusion in the study or regarding CGI scores, meetings were held intermittently in the team to discuss these cases. A consensus decision could therefore be made on diagnosis and CGI rating.

2.8.3 Storage of patient and subject information

Clinical details regarding the patients and personal information of subjects were stored according to protocols for the storage of confidential information. This included the use of filing cabinets either in lockable research offices or in clinical areas which are subject to the provisions for confidentiality. Other patient information, which was part of the normal recording of clinical activity, was stored in the team bases of the psychiatric sectors involved, under usual arrangements for storage of confidential information.

CHAPTER 3

THE EFFECT OF PAROXETINE TREATMENT ON RESPONSES TO META- CHLOROPHENYLPYPERAZINE

3.1 BACKGROUND

In an attempt to understand how the therapeutic effects of serotonin selective re-uptake inhibitors (SSRIs) are mediated, we undertook the present study to assess the effect of a short-term period of treatment with an SSRI, paroxetine, on hormonal, temperature and behavioural responses to m-CPP in healthy volunteers. In animal studies repeated administration of SSRIs produces a number of adaptive changes in both pre- and post-synaptic serotonin receptors (Johnson, 1991). In human studies chronic SSRI treatment has shown a decrease in a number of functional responses to m-CPP, as discussed in Chapter 1 (Kennedy et al, 1993; Kennett et al, 1994a; Maj et al, 1996). As 5-HT_{2C} receptor antagonists have anxiolytic properties in animal models it was hypothesised that this latter action might underlie some of the anxiolytic effects of SSRIs in patients.

In those patients suffering from obsessive compulsive disorder the capacity of m-CPP (taken orally) to produce a worsening of symptoms was abolished by chronic treatment with fluoxetine (an SSRI), and clomipramine (a serotonergic tricyclic antidepressant) (Zohar et al, 1988; Hollander et al, 1991).

This effect could, however, have been due to the clinical benefit derived from treatment. Although the symptomatic deterioration was abolished the hormonal responses to m-CPP were not, but this finding was further complicated by the fact that the m-CPP serum levels were elevated in the second challenge following clomipramine treatment, in the earlier study. This may have been due to the inhibition of m-CPP metabolism in the liver by the antidepressant .

In this study it was therefore hypothesised that the period of treatment with paroxetine would attenuate both prolactin and hyperthermic responses to m-CPP. To avoid possible pharmacokinetic interactions between m-CPP and paroxetine the m-CPP was administered intravenously at a dose known to be well-tolerated. The study was powered to detect a reduction in behavioural effects as a replication of the OCD study quoted above where 6 patients were treated with fluoxetine (Hollander et al, 1991).

3.2 METHODS

3.2.1 Subjects

The study was approved by the local Ethics Committee, the Oxford Psychiatric Research Ethics Committee, and healthy volunteers aged between 18 and 50 years were recruited by local advertisement or from the volunteer bank. At clinical interview before the start of the study it was determined that they had no personal history of mood disorder.

Subjects were excluded if they had a personal medical history of asthma or migraine as m-CPP has been known to precipitate episodes of these conditions. The included subjects were found to be physically healthy by clinical examination and ECG trace.

Blood tests were carried out to evaluate renal, liver, kidney and thyroid function. In addition, it was ascertained, on questioning, that the subjects had not used any psychotropic substances, either medical or illicit, in the three months prior to testing.

Seven subjects were tested (5 males, 2 females), mean age 35.6 years (range 22 to 45 years). They were fully informed regarding the protocol and gave signed informed consent to this study. The 2 female subjects were tested in the early follicular stage of the menstrual cycle (between days 3 and 8), details of which were determined at the initial clinical interview.

3.2.2 Paroxetine treatment

Subjects received paroxetine 20mg daily for one week, at which point the dose was increased to 30mg per day and they remained on the increased dosage for a further 2 weeks. This period was chosen in view of literature showing superior paroxetine response compared to fluoxetine at week three (De Wilde et al, 1993; Geretsegger et al, 1994). One male was unable to continue at 30mg daily in view of dizziness and nausea and the dose was therefore continued at 20mg per day. Other than this individual no significant side effects were described and the drug was tolerated well.

3.2.3 Neuro-endocrine challenge procedure

The subjects attended the unit for blood sampling related to neuro-endocrine measurements on two series of occasions in total, and drug or placebo was administered on a random basis, in a balanced order design. The first series (two single days) took place prior to the subject commencing paroxetine in order to establish base line measures and achieve responses to m-CPP unaffected by anti-depressant medication. The second series of visits (two days) occurred at three weeks after medication had been commenced and either placebo or drug was administered, depending on the order determined by the first test. (See Table 3.1). Neuro-endocrine testing was carried out following an over-night fast with the subjects having been requested not to eat or drink from midnight. They were transported to and from the research unit by taxi.

Table 3.1: Paroxetine Study protocol.

Occasion	Procedure
Visit 1	Assessment, screening, consent, phys. exam.
Session 1 – neuroendocrine challenge test	Placebo or drug administered.
Session 2 – neuroendocrine challenge test	Alternative administered depending on previous session, by randomisation.
Paroxetine commenced	In females commencement was timed for subsequent tests to fall in follicular phase.
Session 3 – neuroendocrine challenge test	Three weeks post paroxetine started. Drug or placebo administered.
Session 4 – neuroendocrine challenge test	Alternative administered depending on previous session, by balanced order.

3.2.3.1 m-CPP dose

The subjects attended for 2 series of paired tests involving a neuro-endocrine challenge, each series consisting of m-CPP or placebo in a double-blind, random order (see table 3.2), cross-over design. The time interval between the two sessions was 1-3 days. The drug or placebo was given at a point in the testing which fell between 9.30 and 10.00 a.m. (time '0'). m-CPP for intra-venous use (Mandeville Medicines Ltd.; Aylesbury; UK) was administered by a dosage schedule to avoid excessive side-effects (Kalus et al, 1992) according to mass (0.05mg per kg) and constituted into a 20ml volume using 0.9% NaCl. It was then administered intravenously over 20 seconds. On the other day 20 ml of 0.9% saline was administered by intravenous injection, as placebo, also over a 20 second period.

Table 3.2: Paroxetine treatment project - study design. Subjects who received m-CPP on the first day of the first series of sessions also received the same order for the second series as randomisation dictated a specific order for the whole study in a balanced order design. (M=m-CPP; P=placebo)

Subject	Test day			
	1	2	3	4
1	P	M	P	M
2	P	M	P	M
3	M	P	M	P
4	P	M	P	M
5	M	P	M	P
6	M	P	M	P
7	P	M	P	M

3.2.3.2 Sampling protocol

Subjects attended the laboratory at 08.30 hours and were cannulated with an indwelling venous catheter which was maintained patent with heparinised saline. Following a 30 minute rest period blood samples were removed for base line endocrine measures and then at 15 minute intervals until one hour had lapsed, post administration of drug or placebo.

m-CPP or placebo was administered 1 hour after cannulation. Following the administration of the intravenous injection via cannula, blood samples were therefore taken at 15 minute intervals, at fifteen minutes and then on 3 further occasions (+15, +30, +45 and +60 minutes post drug or placebo) and thereafter removed at 30 minute intervals (+90, +120 and +150 minutes post drug or placebo).

3.2.4 Monitoring procedures

Subjects were able to relax and read neutral material in between sampling procedures but were not permitted to eat, drink, smoke or watch television. Pulse, blood pressure and oral temperature were measured at base-line and at half-hourly intervals thereafter. Pulse and blood pressure measurements were taken automatically by machine and the oral temperature was recorded using a glass mercury thermometer which remained in place for 10 minutes prior to the recording being made.

3.2.5 Subjective ratings and scales administered

At baseline assessment the clinician involved completed a Hamilton-Depression scale (HAM-D) (Hamilton, 1960) and, at the start of the study, on the first neuro-endocrine test day, subjects completed the Beck Depression Inventory (BDI) (Beck et al, 1961) in order to exclude intercurrent depression. None of the subjects was found to score in the depressed range, prior to paroxetine treatment, for either of these measures, which have been described in more detail in Chapter 2. BDI scores ranged from 0 to 7 pre-treatment (mean = 2.3). Ham-D scores ranged from 0 to 5 (Mean = 2). Subjective ratings were recorded on visual analogue scales (VAS) for the measures “nausea”, “light-headedness”, ‘happiness’ and “anxious” on 10cm scales, as described in Chapter 2. These visual analogue ratings were commenced at time ‘0’ and initially repeated five minutes post-administration of drug or placebo. After this point they paralleled the blood sampling frequency, at 15 minute intervals up to one hour post drug/placebo and thereafter at 30 minute intervals until 150 minutes were completed post administration. Copies of the scales can be seen in appendix 3.

3.2.6 Biochemical assays

Initially 10mls of blood was removed prior to specimen sampling. This was to clear the heparinised saline remaining in the cannula. Following this 2 further blood samples were obtained and stored in ice.

Following the study these two samples were separated by centrifugation and the plasma was stored at -30°C . Prolactin concentrations were determined by a standard immunoradiometric assay, as described in Chapter 2 (reagents provided by Netria, London). The inter- and intra-assay co-efficients of variation over the range encompassed by this standard curve were 4.8% and 2.4% respectively. Plasma m-CPP was measured using HPLC as previously described in Chapter 2 (Odontiadis, 1996). The limit of detection of the assay was 0.2 nanograms per ml, for 1ml of plasma.

3.2.7 Statistics

Plasma m-CPP levels for each subject were calculated as the mean for the plateau phase (30-90 minutes) (Benjamin et al, 1996) and compared by paired t-tests. VAS scores were measured as peak change from base-line and analysed by repeated measures ANOVA in the same way as the prolactin and temperature data.

Base-line PRL tests were also carried out using paired t-tests (2-tailed). Plasma PRL and temperature were plotted against time and calculated as area under the curve (AUC) using the trapezoid rule with subtraction of base-line extrapolated from time "0". The AUC's were then analysed by repeated measures analysis of variance (ANOVA) with two main factors, "treatment" (m-CPP vs placebo) and "occasion" (pre vs post - paroxetine). In addition, AUC responses to placebo were subtracted from those to m-CPP to provide placebo - corrected AUC responses to m-CPP. The values obtained pre and post - paroxetine treatment were compared with paired t-tests.

3.3 RESULTS

3.3.1 Prolactin levels

The AUC PRL data showed a trend main effect of m-CPP ($F=3.09$; $df=1.6$; $P=0.13$) but no significant effect of placebo on ANOVA testing ($F=1.07$; $df=1.6$; $P=0.34$). However, a significant interaction was seen between m-CPP and paroxetine ($F=10.1$; $df=1.6$; $P=0.019$). m-CPP day base-line (time '0') prolactin measures did not differ significantly pre and post paroxetine treatment, 204.83 vs 204.78 mIU/l respectively (t-test; $p=0.99$).

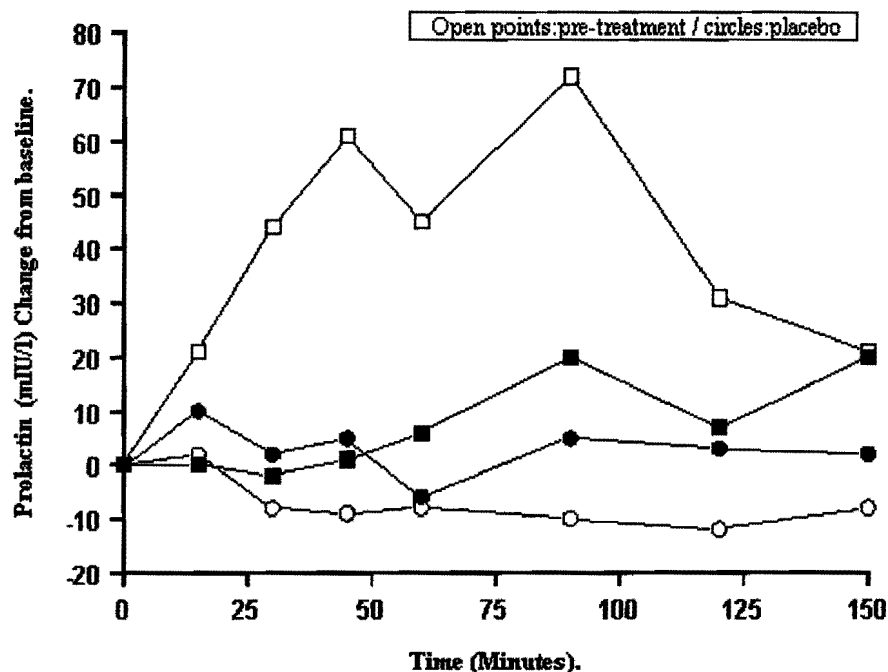


Figure 3.1: Plasma prolactin concentrations (shown as change from base-line in mIU/ml) before and after paroxetine treatment, on m-CPP and placebo days. PRL response to m-CPP was significantly reduced after treatment, in mIUx h/ml (ANOVA; $P=0.007$). Error bars omitted for clarity.

The mean \pm SEM PRL response following placebo did not differ before and after paroxetine treatment (-20 ± 15 vs 7 ± 42 mIU x H/ml; $P=0.65$), but the PRL response to m-CPP was significantly decreased (103 ± 36 vs 12 ± 33 mIU x H/ml; $P=0.007$) (See figure 3.1; SEM points have been omitted for reasons of clarity). The placebo subtracted AUC data further confirmed that the PRL response to m-CPP was significantly less after the period of paroxetine treatment (See Table 3.3).

Table 3.3: Summary of the effect of paroxetine on prolactin responses to m-CPP. The pre and post treatment difference was significant, $P<0.025$, for placebo corrected AUC measures.

Subject	Prolactin AUC (mIUxh/ml)	
	pre-paroxetine	Post paroxetine
1	123	94
2	52	-226
3	180	65
4	5	-31
5	47	-44
6	197	147
7	75	-10
Mean \pm SEM	97\pm27	0.7\pm46

3.3.2 Thermic responses

The temperature data, calculated as AUC, demonstrated a significant main effect of m-CPP ($F=7.4$; $df=1.6$; $P=0.035$) but no main effect of paroxetine was seen ($F=3.49$; $df=1.6$; $P=0.11$). However, a significant interaction between m-CPP and paroxetine was seen ($F=6.54$; $df=1.6$; $P=0.043$).

It is therefore clear that the usually expected positive effect on temperature was seen following m-CPP administration but that the extent of the increase has been diminished by the period of treatment on paroxetine. This can be seen graphically in Figure 3.2 (SEM error bars have been omitted for reasons of clarity).

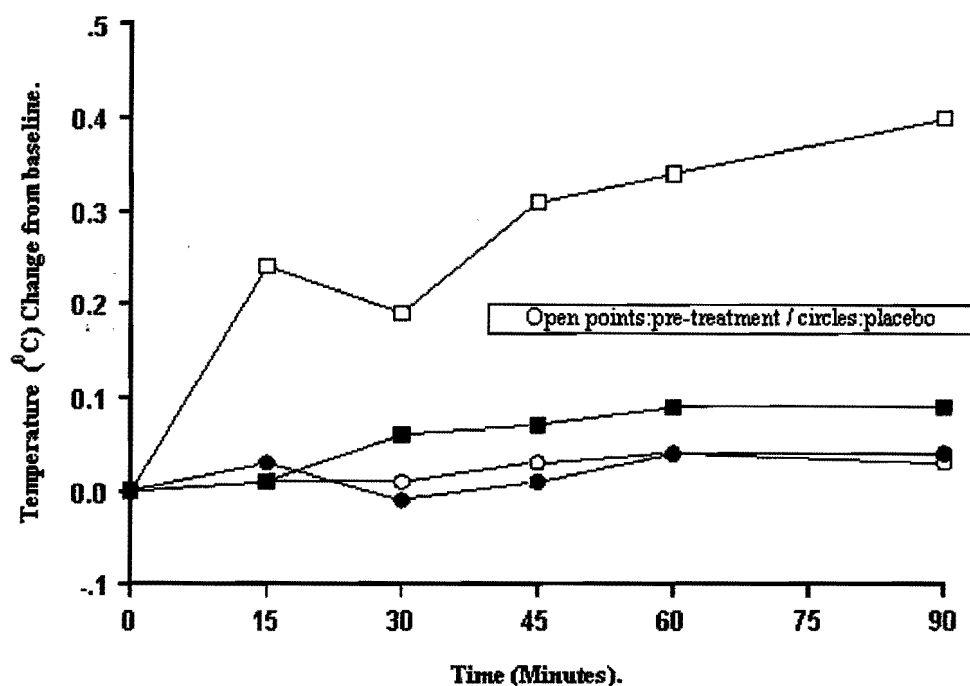


Figure 3.2: Comparison of temperature change ($^{\circ}$ C) before and after paroxetine treatment on placebo and m-CPP days. Temperature elevation is significantly reduced following m-CPP administration after paroxetine treatment (ANOVA; $P=0.031$).

Further testing, using the mean AUC data, showed that the mean \pm SEM temperature response following placebo did not differ before and after paroxetine (2.3 ± 5 vs $2.8 \pm 7.5^{\circ}\text{C} \times \text{min}$; $P=0.96$) but the temperature response to m-CPP was significantly reduced (38.5 ± 8 vs $8.1 \pm 12^{\circ}\text{C} \times \text{min}$; $P=0.031$).

The placebo-corrected AUC data also showed that the hyperthermic effect of m-CPP was significantly less after paroxetine treatment (See Table 3.4).

Table 3.4. Summary of the effect of paroxetine on thermic responses to m-CPP. The pre and post treatment m-CPP induced difference was significant for placebo-corrected AUC measures ($P < 0.05$).

Subject	Temperature:- placebo corrected AUC (°C x min)	
	pre-paroxetine	post paroxetine
1	20	20
2	90	17
3	47	21
4	27	-30
5	32	-26
6	90	17
7	29	37.5
Mean ± SEM	36.6±9.8	5.4±9.7

3.3.3 Visual analogue ratings

The following graphs detail the results of the visual analogue scores as discussed previously in the methods section (Chapter 2).

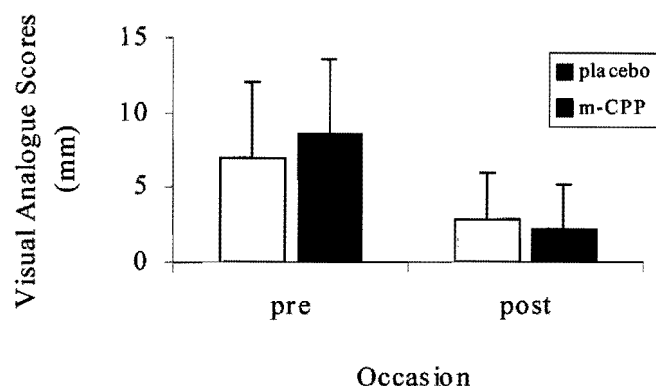


Figure 3.3: Nausea – visual analogue ratings (mm) before and after paroxetine treatment ($P > 0.05$, paired t-test).

The ANOVA of the VAS data for “nausea” and “anxious” showed no significant main or interactive effects of m-CPP and paroxetine (Figures 3.3 and 3.4). However, six out of seven subjects showed a reduction in mean VAS scores of anxiety on the placebo day after the three week period of paroxetine treatment.

In a similar manner, six out of seven subjects (a different group) showed a non-significant reduction in mean anxiety scores on the m-CPP occasions after paroxetine treatment, supporting a general anxiolytic effect of the anti-depressant. Although it has been previously reported that elevation of anxiety levels occurs following the administration of m-CPP, it is evident from the self-report (VAS) anxiety measures recorded in Figure 3.4 that although there is a suggestion of a small positive effect, there was no significant anxiogenic effect of this dose of m-CPP.

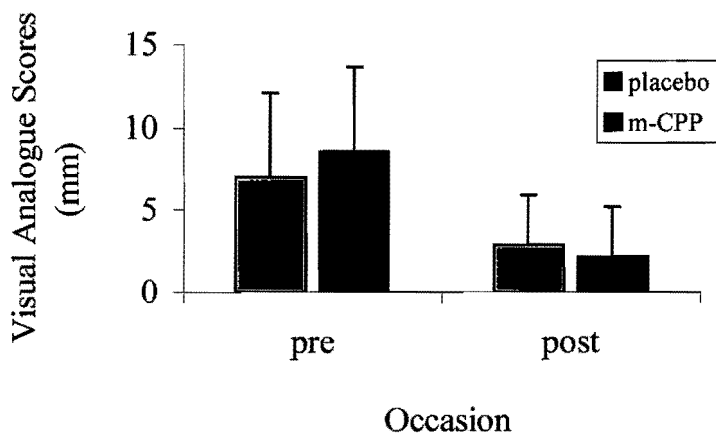


Figure 3.4: Anxiety - visual analogue ratings (mm) before and after paroxetine treatment ($P > 0.05$, paired t-test).

As might be expected, the “light-headed” data showed a significant main effect of m-CPP (F=7.66; df=1.6; P=0.032) but no main effect of paroxetine (F=0.217; df=1.6; P=0.22) or paroxetine by m-CPP interaction (F=3.26; df=1.6; P=0.12) (Figure 3.5).

Peak ratings of light-headedness in response to m-CPP were lower after paroxetine treatment than before, but this was not statistically significant (Pre vs post paroxetine: 24.3 ± 8.4 vs 10.0 ± 4.4 ; P=0.11, paired t-test).

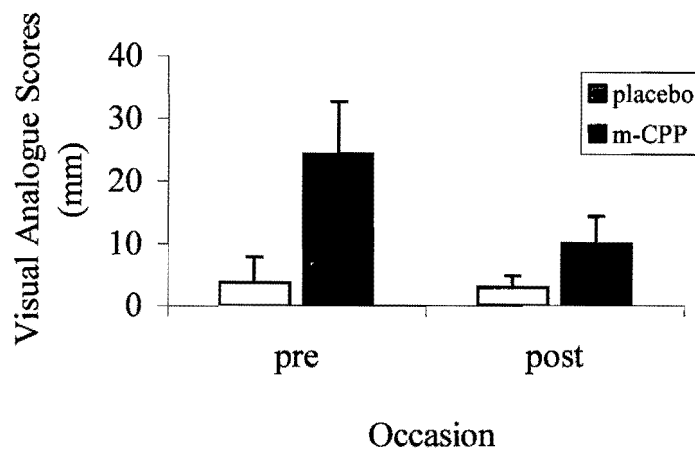


Figure 3.5: Light headedness. Visual analogue ratings (mm) before and after paroxetine treatment (P=0.11, peak value post-hoc paired t-test).

3.3.4 Drug levels

3.3.4.1 m-CPP levels

Although an increase can be seen in the m-CPP levels following the period of paroxetine treatment, in Figure 3.6, the mean plateau levels of m-CPP after the intravenous injection did not differ significantly when compared statistically by paired t-tests ($p=0.325$).

Table 3.5: Summary of the variation in plasma m-CPP levels in ng/ml, before and after paroxetine treatment ($P>0.05$; NS).

Subject	Plasma m-CPP level (ng/ml)	
	Pre-paroxetine	Post paroxetine
1	58.7	41.2
2	13.3	30.1
3	41.9	42.3
4	20.4	28.4
5	23.6	43.5
6	52.5	18.1
7	Missing	Missing
Mean \pm SEM	33.9\pm7.5	35.1\pm 4.1

As i.v administration avoids first pass metabolism, it is unlikely that this non-significant increase could have been caused by inhibition of the hepatic metabolism of m-CPP by paroxetine, as discussed in the introduction, regarding the study by Hollander and team (1991).

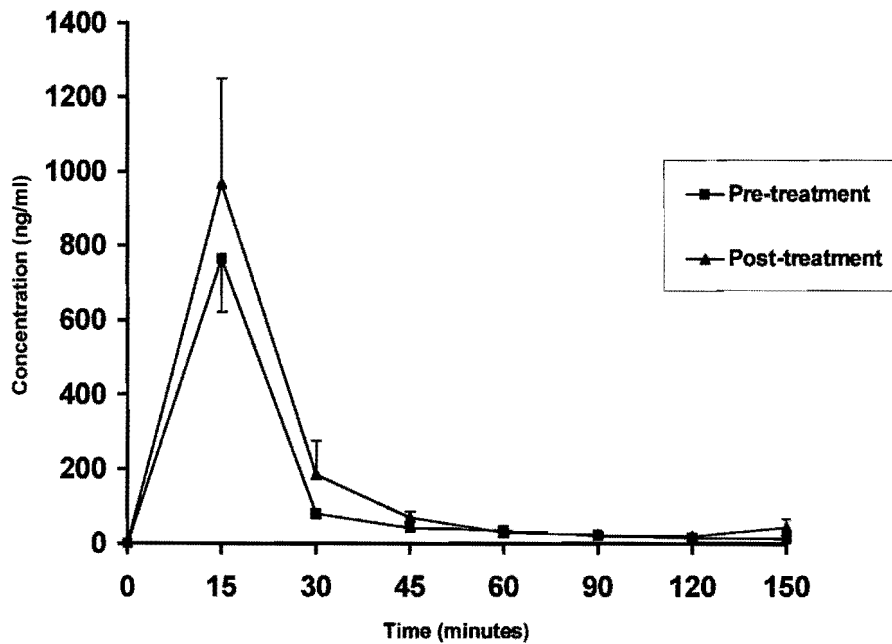


Figure 3.6: Plasma m-CPP drug levels in ng/ml (shown as mean \pm SEM) measured before and after paroxetine treatment in seven healthy subjects. The values are calculated by measuring the mean levels from the plateau phase of the neuro-endocrine tests, from +30 minutes to +90 minutes (Not significant by paired Student's t-test ; $p=0.375$).

3.3.4.2 Paroxetine levels

Blood samples taken at the final neuro-endocrine challenge session confirmed that the subjects had all been compliant with their paroxetine however there was wide variability in the levels and the doses were not modified according to BMI or previous level testing as it is not usual for these to be used in clinical practice (See Figure 3.7).

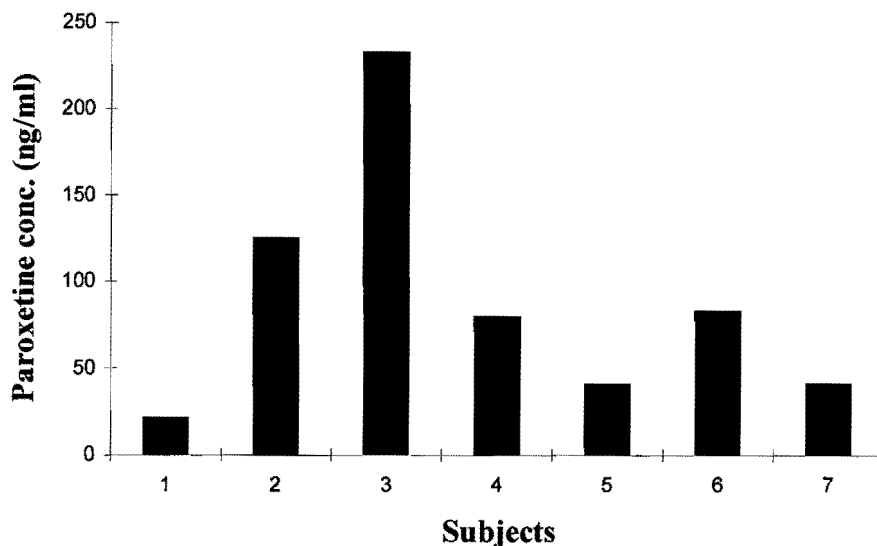


Figure 3.7: Plasma paroxetine drug concentrations in ng/ml at 3 weeks treatment in seven healthy volunteer subjects (plateau phase: +30mins to +90 mins) on final day of testing.

3.4 DISCUSSION

The major findings of this study are compatible with other work suggesting that SSRI treatment down-regulates the sensitivity of post-synaptic 5HT_{2C} receptors. This was seen following treatment of healthy subjects with a lowering of the prolactin and hyperthermic response to m-CPP in the neuroendocrine test condition. It is unlikely that drug side effects of m-CPP could have contributed in a major way to these findings through other non-specific physiological effects. Although the visual analogue scale scores concerning light-headedness were reduced following the three week treatment period, this was not in a statistically significant manner.

It is, however, necessary to consider alternative explanations for the above findings. As was discussed previously, m-CPP has activity at other serotonin receptor sites although its major agonistic effect is at the 5HT_{2C} receptor site. The 5HT_{1A} receptor has also been implicated in prolactin release in humans (Anderson et al, 1990). This finding has been supported by the observed antagonism towards m-CPP of pindolol when monitoring prolactin response, supporting the notion that 5HT_{1A} receptors may play a role in m-CPP induced prolactin release (Meltzer and Maes, 1995).

A further possibility involving the 5HT_{1A} receptors in explanation of the above findings is that paroxetine treatment blunts the endocrine responses to 5HT_{1A} receptor agonists including gepirone (Sargent et al, 1997) and this could also account for the reduced prolactin response to m-CPP in this study. It is less likely that this could explain the reduced hyperthermic response as 5HT_{1A} receptor activation usually leads to a decrease rather than an increase in body temperature (Anderson et al, 1990).

There is also some evidence from animal studies involving in-vivo microdialysis that m-CPP can promote 5-HT release from presynaptic terminals (Baumann et al, 1993). This may suggest that the prolactin response to m-CPP could also involve indirect activation of post-synaptic 5-HT receptors. Other animal work has suggested that this effect can be blocked by SSRIs, possibly by the interference of SSRIs with the action of the releasing substance on the 5-HT transporter. In another study fluoxetine reduced the prolactin response to m-CPP in rats. Thus it is conceivable that paroxetine might prevent the endocrine and thermic effects of m-CPP by preventing m-CPP-induced 5-HT release.

Against this is the finding in animal studies that the release of prolactin after m-CPP administration is enhanced following destruction of serotonin neurones by 5-7 DHT. This suggests that m-CPP - induced prolactin release is indeed mediated by post-synaptic 5-HT receptors (Quattrone et al, 1981).

An alternative possibility for the effect of paroxetine to diminish the functional effects of m-CPP is that paroxetine may possess direct 5-HT_{2C} receptor antagonist properties. However, this possibility is not supported by binding data as paroxetine has a lower affinity for 5HT_{2C} receptors than fluoxetine (Wong et al, 1991).

As regards the design of the study, it was not possible to control for order effects in the administration of m-CPP and placebo. It is therefore possible that the reduced responses represent a loss of reactivity in the second test. This has been found in other studies where intravenous injections of m-CPP induced tolerance to a number of its expected effects (Benjamin et al, 1996). This is less likely, however, in view of the time gap separating the two studies, which was three weeks. It has also been found previously, in a cross-over design study using intravenous m-CPP, that there was no evidence of an order effect on the hormonal and behavioural responses to m-CPP (Silverstone and Cowen, 1994). Other studies mentioned previously, utilising clomipramine and fluoxetine in OCD, did not find reduced endocrine responses to m-CPP but, as discussed previously, this may have been due to the increased levels of plasma m-CPP (Hollander et al, 1991; Zohar et al, 1988). Although powered as in the Hollander study, to detect behavioural effects, it may be that the sample size was too low to elicit psychological variations post treatment.

In spite of the absence of reduced endocrine responses to m-CPP in the above studies, it was noted that clomipramine treatment reduced m-CPP stimulated hyperthermia (Zohar et al, 1988). In our study plasma levels of m-CPP were not significantly increased following paroxetine treatment. This is most likely due to the use of an intravenous mode of administration. The dose of m-CPP used in our study was also chosen to be well tolerated, and endocrine responses were therefore sub-maximal. Should the above findings in our study be correct then the mechanisms involved in the reduction of 5-HT_{2C} receptor sensitivity are not clear. Both 5-HT_{2C} receptor changes or receptor transduction mechanisms could be involved

3.5 CONCLUSIONS

5-HT_{2C} receptor down-regulation has been suggested to relate to the function of SSRIs in reducing anxiety in patients as well as in the treatment of OCD. While drugs with 5-HT_{2C} receptor antagonist properties may be effective in anxiety they do not appear to be effective in OCD .

Indeed, 5-HT_{2C} receptor antagonists such as ritanserin and metergoline can undermine the effect of SSRIs in OCD. Perhaps SSRIs increase 5-HT neurotransmission at 5-HT_{2C} receptors and the down-regulation of post-synaptic 5-HT_{2C} receptors occurs in response to this.

The role of 5-HT_{2C} receptors in the treatment of depression is less clear. Some antidepressants such as mirtazapine and nefazodone are effective antidepressants but many antidepressants have no activity at the 5-HT_{2C} receptor. It has also been noted that desipramine fails to reduce m-CPP stimulated behaviours in animals and it is not effective in the treatment of OCD (Kennett et al, 1994). This question can be better answered with the use of selective 5-HT_{2C} receptor antagonists and will increasingly continue to be investigated using molecular techniques in view of findings suggesting that antidepressants not only act pharmacologically but physiologically. However molecular studies thus far have shown an increase in 5-HT_{2C} m-RNA in the rat brain following imipramine treatment rather than a decrease, which might therefore imply an attempt to boost receptor numbers in the face of antagonism towards the receptors (Tohda and Watanabe, 1996).

Further research possibilities include the studying of the time course during which SSRI treatment reduces the endocrine and thermic responses to m-CPP, as a delayed effect would suggest receptor desensitisation to a greater extent than a direct receptor effect or inhibition of pre-synaptic 5-HT release.

CHAPTER 4

THE EFFECT OF ALLELIC VARIATION ON RESPONSE TO SEROTONIN SELECTIVE RE-UPTAKE INHIBITORS.

4.1 BACKGROUND

With the discovery of different polymorphic versions of the serotonin receptors and the serotonin transporter it has become a clinically relevant question as to whether polymorphic variation might lead to differential response rates in patients suffering from depression when treated with SSRIs. The details of these polymorphic variants were described in Chapter 1.

The aim of the present study was therefore to establish whether the cys23ser polymorphic variation in the 5-HT_{2C} receptor gene or the 5-HTTLPR variant might predict a greater response of patients to SSRIs and the serotonergic tricyclic antidepressant, clomipramine.

Initial work has focussed on two polymorphisms linked with the serotonin transporter (5-HTT) gene – one in the promoter region (5-HTTLPR) and another in intron 2 (Stin2).

Several studies have detected allelic association between this gene and clinical response to SSRIs. In one study patients with the long polymorphic variant - 'l/l' and heterozygotes with the short and long - 's/l' - versions of the promoter region polymorphism were more likely to show an improved response to fluvoxamine, an effect abolished in the group also treated with pindolol (Smeraldi et al, 1998).

In two similar studies utilising paroxetine, an increased response rate was seen in subjects with at least one copy of the long variant of the 5-HTTLPR polymorphism in a study involving young adults (Zanardi et al, 2000) and in a further study involving an older group of patients. In the latter study a more rapid response was seen for those patients homozygous for 'l/l' promoter alleles compared to those with an 's' allele (heterozygotes or homozygotes) (Pollock et al , 2000). However, an enhanced clinical response has also been reported in patients homozygous for the short version - 's/s' - of the 5-HTTLPR polymorphism, and in homozygotes with the Stin2.12 version of the intron 2 polymorphism (Kim et al, 2000). In a related study in Obsessive Compulsive Disorder, no difference was seen between the two groups when both polymorphisms were examined, in considering their response to fluoxetine or clomipramine (Billett et al, 1997). Should it be ascertained that polymorphic variation is associated with differing relative response rates to different types of anti-depressant medication, this would provide the clinical option of genotyping, in advance of antidepressants being used, in order to facilitate an optimal pharmacogenetic choice of treatment. Although the sample size in this study is small for a genetic study it is powered to detect a minimum difference of 48% of responders with a serine allele if the non-responder serine rate were to remain constant at 33.3%.

Data suggest that pharmacogenetics can be used to predict response to clozapine treatment in schizophrenia since 19 polymorphisms have been shown to predict response to the atypical anti-psychotic, clozapine, with a 76,7 % rate of success i.e. a positive predictive value of 0.76 and a negative predictive value of 0.82, in a test of one model utilising algorithms (Arranz et al, 2000).

In view of the potential of the above strategy and in order to extend the framework which arose from an initial paper showing a correlation of the serine substituted 5HT_{2C} polymorphism with response to clozapine (Sodhi et al., 1995), it was decided to investigate whether response to serotonergic antidepressant medication might also correlate with this 5-HT_{2C} receptor polymorphic variation.

Additionally, in view of the more recent interest in the promoter region variants of the serotonin transporter gene and the pivotal role of the transporter in the most widely accepted model of SSRI function, the 5-HTTLPR polymorphism was also investigated in the study.

Related work on a healthy volunteer sample, from which subjects with specific serotonin transporter and 5-HT_{2C} polymorphisms had been recruited for the studies described in Chapters 5 and 6, permitted the comparison of polymorphism rates in the depressive sample with rates in a control group who had described no current self-reported or previous psychiatric history.

4.2 METHODS

4.2.1 Recruitment of groups studied

4.2.1.1 Patient recruitment

Patients were recruited from: a) the database of the Psychopharmacology Research Unit, where they had previously been involved in treatment studies of major depression.

b) directly from a number of general practices in the Oxfordshire area and, c) from a group of patients who had taken part in research studies on major depression according to the protocols of the Hertfordshire Neurosciences Research Group.

Ethical approval for the study was obtained from the local Ethics Committee (OPREC).

Patients were aged between 18 and 65 and specific exclusion criteria included:-

- presence of an organic disorder.
- significant illicit substance misuse.
- co-morbidity for severe personality disorder.

The patients were contacted either by telephone or by letter to invite them to participate in the study or they were referred by local GP's for a psychiatric consultation and further management. At the initial interview it was established whether they had a DSM3-R/DSMIV diagnosis of major depression (Axis 1 diagnosis).

For the patients who had previously been managed in the unit the diagnosis of DSM3-R major depression had already been made for previous studies. For those attending the unit, the diagnosis was confirmed at clinical interview or, if unable to attend, a case note diagnosis was made according to DSM3-R/DSMIV criteria. Patients were invited to contribute a buccal swab or a blood sample, depending on their preference. Some patients preferred not to attend the unit but were willing to send a buccal swab by post. Should further information have been lacking regarding their clinical progress, this was obtained by letter, or by telephone.

All patients who attended the unit gave written, informed consent, however the Ethics Committee had agreed that those patients sending a buccal swab by post would be giving implicit consent and that a separate form was therefore unnecessary. They had received an information sheet and patients sending swabs by post had also received a telephone call to discuss the study.

The patients were or had been under the treatment of psychiatrists within the department and it was therefore possible to establish consensus diagnoses in situations where the diagnosis was not clear, at meetings convened for this purpose.

4.2.1.2 Control group

The control group consisted of subjects who had attended blood donor clinics in Oxfordshire, as described in Chapter 1.

They gave informed consent to provide a cheek swab for DNA analysis and also completed a questionnaire with demographic information as well as personal details regarding the presence or absence of mental health problems, past and present.

4.2.2 Genotyping

This was carried out according to the techniques described in Chapter 1. Samples taken at the Psychopharmacology Unit or received by mail were frozen at -70 degrees prior to genotyping. Those samples taken at Queen Elizabeth II Hospital, Hertfordshire, were frozen initially in a freezer box at -5 degrees and then transferred to the -70 degree freezer once local collection had been completed.

4.2.2.1 5-HT_{2C} receptor polymorphisms

The polymorphism used to determine relative response rates, according to the 5-HT_{2C} gene was the serine/cysteine substitution at codon 23 (there is a G to C change at nucleotide 68).

As discussed in chapter 1, the serine substituted version is found in approximately 13 % of the population (Sodhi et al, 1995) but, in view of X-linkage, females may be homo- or heterozygotic while all males are hemizygous (Lappalainen et al, 1995).

4.2.2.2 5-HT Transporter polymorphism

The polymorphism used to determine whether allelic variation in the transporter gene may be relevant to SSRI response is a variant derived from the promoter region of the human serotonin transporter gene (5-HTTLPR). This is a 44-base-pair insertion/deletion polymorphism which gives rise to a short (s) and long (l) form of the gene. The characteristics of this gene and its effects have been described in Chapter 1.

4.2.3 Clinical response

4.2.3.1 Clinical Global Impression Scale

Sources of information regarding patient clinical response to serotonergic medication included the clinical case notes, which recorded details derived from consultations of the Psychopharmacology Unit medical staff with the patients, rating scales regarding entry to the studies and progress scores on those patients taking part in clinical research studies, and information received from general practitioners at either referral or by subsequent request, where information was lacking regarding response. As variability was evident in the quantity and type of data collected from the patients, relating to whether they had been involved in other studies or referred by G.P's, all of the available information was collated and a single clinical global impression (CGI) score was derived (See Table 2.5).

In each case, where possible, the treating clinician derived the score and completed a questionnaire regarding demographic details, age of onset, diagnosis, comorbidity, past history of treatment, details of response and past history of self harm. The clinicians were blind to the patients' 5-HT_{2C} and 5-HTTLPR genotypes at the time of the decision regarding clinical response. For those patients recruited by the Hertfordshire Group, MADRS scores were available to the treating clinicians regarding response to paroxetine and CGI scores were derived according to common criteria with the Oxford patients.

4.2.3.2 Consensus ratings

For the derivation of CGI scores from those patients where the involved clinician had left the unit, information was initially collated from all of the clinical records. Where necessary, this included a confirmation of diagnosis according to DSM-3R/DSMIV criteria. For a number of these patients senior clinical academic staff had also been involved in decisions regarding the patient's treatment and in many cases, had met the patients themselves.

For the above patients and in any other situations where either the diagnosis or clinical response to treatment was in question, the issues were considered at consensus meetings of the clinicians involved with the study. In this way consensus opinions were recorded regarding unclear diagnosis or response. The clinicians involved in these meetings were all blind to gene subtype.

4.2.4 Statistics

Analyses regarding the serotonin transporter gene were different to those carried out concerning the 5-HT_{2C} polymorphism, as the X-linkage of the latter gene necessitated separate analyses for males and females.

Patients were grouped into 'responders' by grouping those with CGI=1 and 2 (good and marked) and 'non-responders' which referred to CGI=3 and 4 (poor and deterioration). Chi squared analyses (Mantel Haenszel) were performed on the data dichotomised as above and by division of the gene groupings according to differing assumptions regarding the relevance of the effect of a specific allele. In addition to the chi-squared analyses, tests of linear association were carried out using the CGI score as one variable and incremental quantities of gene dose as the other.

For the serine dominant model greater power was achieved by combining the 5-HT_{2C} receptor gene cys/ser patients with the serine homozygote group in view of the limited numbers of serine homozygotes. In the comparison of 5-HTTLPR polymorphisms both short (s) allele dominant and recessive models were used, as previous studies had demonstrated both an increased occurrence rate of this polymorphism in depressed samples (unipolar and bipolar) over healthy control populations (Collier et al, 1996a) and no association (Kunugi et al, 1997). In the comparisons of 5-HT_{2C} and 5-HTTLPR polymorphism rates between the depressive group and the healthy control group, X² analyses were used.

4.3 RESULTS

4.3.1 General features of the samples

4.3.1.1 Depression group

Of a total group of 416 patients who had been treated or had taken part in research studies at the Psychopharmacology Unit between 1995 and 2000, it was possible to obtain blood samples or a buccal swab on 188 patients who had been treated on SSRIs or clomipramine (110 females and 78 males), and who satisfied criteria for major depression (DSM3R/4).

A total of 17 patients were recruited from the Hertfordshire group. As unipolar depressives were over-represented in the target patient group only 6.3% of the final sample was diagnosed as suffering from bipolar disorder.

The age of the patients was 45.2 ± 1.3 years (mean \pm SEM) and the range was 21-70 years. Seven patients were excluded to ensure that age related effects relating to differential diagnosis were minimised. As a result 181 patients were aged 21 to 65 years. Of this sample, four individuals had a recorded non-Caucasian ethnicity and were therefore excluded from further analyses in view of the possibility that differences in the ratio's of polymorphisms between ethnic groups could confound the analyses. The final sample size for the main analyses was therefore 177 patients. Of these 71 were male and 106 female patients. In view of the small number of patients recruited from the Hertfordshire group and the small percentage of bipolar patients their results were not analysed separately.

4.3.1.2 Control group

There were 328 subjects in total, 138 were male and 190 were female (mean age \pm SEM is 38 ± 13 years; range: 19-71 years). All of the above subjects were Caucasian and their parents and grand-parents were born in the UK.

4.3.2 Demography of response

Of the total sample, 104 out of 177 patients were classed as responders (CGI=1 or 2), which equates to 58.8 %, an average response rate to SSRI anti-depressant medication (Edwards and Anderson, 1999). This included 42 of 71 males (59.2 %) and 62 of 106 females (58.5 %). Dividing the total sample into two groups around the median age (47 years) reveals no age effect in response.

Dividing the group by the possible four CGI scores shows 19.2 % to have been marked or excellent responders, 39.5 % were good or moderately good responders, 27.7% were poor or minor responders and 13.6 % showed evidence of deterioration or no change. It is therefore possible to group all those who showed any measure of response (86.4%), in order to derive a total figure but this would not equate to measures used in clinical studies or in the meta-analyses of response mentioned above, where more stringent response criteria are applied.

4.3.3 5-HT_{2C} receptor polymorphisms

Of the sample of 177 patients, 120 had sufficient blood or buccal swab material available for extraction and identification of the 5-HT_{2C} variants. This included 46 males and 74 females.

In the total sample, polymorphism rates were as seen in Table 4.1 (below). Of the males, 12 (26.1 %) therefore had serine alleles and 34 (73.9%) cysteine alleles. In the female group 3 (4.0 %) patients were homozygotic for serine, 21 (28.4 %) were heterozygotes, and 50 (67.6 %) were cysteine homozygotes.

Table 4.1: Total sample of 120 patients divided by gender and 5-HT_{2C} genotype. The genotyping for the females is in Hardy-Weinberg equilibrium ($\chi^2=0.18$, 1df, NS). The males cannot be tested for Hardy-Weinberg equilibrium because they are hemizygous.

Genotype	Males (%)	Females (%)	Total (%)
Ser	12 (26)	0	12 (10)
Ser/Ser	0	3 (4)	3 (2.5)
Cys/Ser	0	21 (28)	21 (17.6)
Cys	34 (74)	50 (68)	84 (69.9)
Total	46 (100)	74 (100)	120 (100)

4.3.3.1 Gene frequencies in response

The genotyped sample had a slightly lower proportion of responders than the whole patient sample with 65 patient responders out of a total of 120 (54,2 %). Of the responder group, 32.3% had at least one serine allele compared with 27.3% of the non-responder group, a non-significant difference (Mantel Haenszel, $X^2 = 0.36$; 1 d.f.; $p=0.55$). Findings relating to both patient and control groups for the 5-HT_{2C} receptor are presented in Table 4.2.

Findings for the serine homozygotes and heterozygotes versus cysteine homozygotes in a serine dominant model can be seen in Table 4.3, while the findings for the serine recessive model are seen in Table 4.4. Neither grouping is statistically significant.

Table 4.2: Summary of frequencies of 5-HT_{2C} genotypes and alleles in depressed patients and controls.

Genotype	Responders			Non responders			All Patients			Controls		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total
Cys/cys or cys	18	26	44	16	24	40	34	50	84	112	136	248
Cys/ser	0	12	12	0	9	9	0	21	21	0	51	51
Ser/ser or ser	7	2	9	5	1	6	12	3	15	26	4	30
TOTAL	25	40	65	21	34	55	46	74	120	138	191	329
Cys allele	18	64	82	16	57	73	34	121	155	112	323	435
Ser allele	7	16	23	5	11	16	12	27	39	26	59	85

Table 4.3: Comparison of response rates in patients according to the presence or absence of any serine alleles in a serine dominant model; (Mantel Haenszel; $X^2 = 0.36$; d.f=1; p=0.55; NS).

Genotype	Non-responders (%)	Responders (%)	Total (%)
C/S, S or S/S	15 (27.27)	21 (32.31)	36 (30)
C/C or C	40 (72.73)	44 (67.69)	84 (70)
Total	55 (100)	65 (100)	120 (100)

Table 4.4: Comparison of response rates in patients according to the presence or absence of any cysteine alleles in a serine recessive model; (Mantel Haenszel, $X^2 = 0.23$; d.f=1; p=0.63; NS).

Genotype	Non-responders (%)	Responders (%)	Total (%)
S or S/S	6 (10.9)	9 (13.8)	15 (12.5)
C/C; C/S, or C	49 (89.1)	56 (86.2)	105 (87.5)
Total	55 (100)	65 (100)	120 (100)

4.3.3.2 Allele frequencies

The patient group allele frequencies can be seen in Table 4.5 and the normal control group frequencies can be seen in Table 4.6. The frequencies are calculated by adding the number of allele subtypes in each group by gender to give a total group allele score. As males only possess one 'X' chromosome, they either possess a single serine or cysteine allele and are therefore counted once each whereas females, with two, are homo- or heterozygotic.

Table 4.5: Allele rates in the patient sample. Division by polymorphism and gender. Difference is not statistically significant. X^2 (Mantel Haenszel) = 1.34; d.f.=1; p=0.25.

Alleles	Males (%)	Females (%)	Total (%)
Ser allele	12 (26.1)	27 (18.2)	39 (20.1)
Cys allele	34 (73.9)	121 (81.8)	155 (79.9)
Total	46 (100)	148 (100)	194 (100)

The main difference in the allele frequencies seen in the patient sample compared to the normal subject sample is in the slight excess of male patients with serine alleles. This finding is also inflated in comparison with other reports of rates in large normal populations (Lappalainen et al, 1995). The allele rates for the comparative normal control group are shown in Table 4.6.

Table 4.6: Allele rates in the control sample. Division by polymorphism and gender. Difference is not statistically significant. X^2 (Mantel Haenszel) = 0.85; d.f.=1; p=0.36.

Alleles	Males (%)	Females (%)	Total (%)
Ser allele	26 (18.8)	59 (15.5)	85 (16.4)
Cys allele	112 (81.2)	323 (84.5)	435 (83.6)
Total	138 (100)	382 (100)	520 (100)

The male 5-HT_{2C} serine excess contributes to the finding of a serine excess in the patient versus control group seen in Table 4.7, but the difference is not at a statistically significant level.

Table 4.7: Comparison of frequencies of 5-HT_{2C} alleles in normal controls and depressed patients. Difference is not significantly different. X^2 (Mantel Haenszel) = 1.39; d.f.=1.; p=0.24.

Allele	Normal subjects (%)	Depressed patients (%)
Ser allele frequency	85 (16.4)	39 (20.1)
Cys allele frequency	435 (83.6)	155 (79.9)
Total	520 (100)	194 (100)

4.3.4 Serotonin Transporter polymorphisms

Of the sample of 177 patients, 129 had sufficient material for DNA extraction and genotyping. Rates of 5-HTTLPR polymorphisms were as follows: (See Table 4.8).

Table 4.8: Serotonin transporter promotor polymorphism genotypes in patients. Both groups were in Hardy Weinberg equilibrium (males X^2 =0.18, d.f.=1, NS; females X^2 =1.14, d.f.=1, NS).

Genotypes	Males (%)	Females (%)	Total (%)
s/s	12 (23.5)	15 (19.2)	27 (20.9)
s/l	27 (53.0)	33 (42.3)	60 (46.5)
l/l	12 (23.5)	30 (38.5)	42 (32.6)
Total	51 (100)	78 (100)	129 (100)

Although differences are seen in the frequencies of genotypes between males and females, these differences are not statistically significant ($X^2 = 3.13$; d.f.=2; NS).

4.3.4.1 Gene frequencies in response

The CGI score was used to group patients into responders and non-responders as in the analyses performed in relationship to the 5-HT_{2C} polymorphisms (responders are CGI = 1 and 2; non-responders are CGI = 3 and 4). A summary of the results can be seen in Table 4.9.

Table 4.9: Summary of frequencies of 5-HTTLPR genotypes and alleles in depressed patients and controls.

Genotype	Responders			Non responders			All Patients			Controls
	M	F	Total	M	F	Total	M	F	Total	Total
L/L	5	17	22	7	13	20	12	30	42	100
L/S	17	19	36	10	14	24	27	33	60	160
S/S	6	7	13	6	8	14	12	15	27	65
TOTAL	28	43	71	23	35	58	51	78	129	325
L allele	27	53	80	24	40	64	51	93	144	360
S allele	29	33	62	22	30	52	51	63	114	290

Response can be considered by division of the patient sample into two possible groups for analysis, by inclusion of the heterozygotes with either the 'l/l' or 's/s' group of homozygotes (See Table 4.10).

When adopting a model where the short allele is dominant, no significant differences are seen in the rates of polymorphism between the responders and non-responders even though there are relatively more responders with at least one short allele (69.0% vs 65.5%).

Table 4.10: Comparison of rates of response to anti-depressants by presence or absence of a short variant (s) in a short allele dominant model. X^2 (Mantel Haenszel) = 0.18; d.f=1; p=0.67; NS.

Genes	Non-responders(%)	Responders (%)	Total (%)
s/l or s/s	38 (65.5)	49 (69.0)	87 (67.4)
ll	20 (34.5)	22 (31.0)	42 (32.6)
Total	58 (100)	71 (100)	129 (100)

Neither model is statistically significant, however the short allele recessive model exhibits a greater difference between responders and non-responders.

Table 4.11: Comparison of rates of response to anti-depressants by presence or absence of a long variant (l) in a short allele recessive model. X^2 (Mantel Haenszel) = 0.65; d.f=1; p=0.42; NS.

Genes	Non-responders(%)	Responders (%)	Total (%)
S/L or LL	44 (75.9)	58 (81.7)	102 (79.1)
S/S	14 (24.1)	13 (18.3)	27 (20.9)
Total	58 (100)	71 (100)	129 (100)

4.3.4.2 Allele frequencies

As the serotonin transporter gene is not X-linked, two alleles are counted for each individual (See Table 4.12), both males and females.

Table 4.12: Frequencies of Serotonin Transporter promoter region allele distribution in depressed patients by gender. Difference is not statistically significant. X^2 (Mantel Haenszel) = 2.3; d.f.=1; p=0.13.

Alleles	Males (%)	Females (%)	Total (%)
S allele	51 (50)	63 (40.4)	114 (44.2)
I allele	51 (50)	93 (59.6)	144 (55.8)
Total	102 (100)	156 (100)	258 (100)

As can be seen in Table 4.13, we did not replicate the occasionally seen excess of 5-HTTLPR short allele variants in case control allelic association studies of depression (Collier et al, 1996a).

In the latter Collier study three centres all demonstrated higher rates of the short variant compared to controls but the individual centre rates failed to reach significance. However, when pooling their results, a stratified analysis gave a significant overall odds ratio of 1.23 (95% CI: 1.02-1.49; p=0.03) and the homozygous short allele group in the patient sample showed an even higher odds ratio, 1.53 (95% CI: 1.04-2.23; p=0.02).

Table 4.13: Comparison of frequencies of Serotonin Transporter promoter region alleles in normal controls and depressed patients. $X^2 = 1.97$; d.f.= 1; $p=0.16$.

Allele	Normal subjects (%)	Depressed patients (%)
S allele frequency	290 (44.6)	114 (44.1)
L allele frequency	360 (55.4)	144 (55.9)
Total	650 (100)	258 (100)

4.4 DISCUSSION

The previous literature is contradictory, as discussed in the introduction, with different outcomes in the studies due to likely under or over-activity of the transporter variants. Although it is theoretically predicted that the *l/l* polymorphism is more likely to correlate with improved response, as more of the studies have shown this correlation, in our sample no directional effect was seen. It is also important to note that the decisions regarding response were retrospective. Possible reasons for the lack of a significant effect need to be considered. Although the patients with known non-caucasian ethnicity were excluded from the study, the ethnic origin of a number of patients was not recorded by the clinician and so for 28 patients ethnicity was assumed as caucasian. Exclusion of these cases did not change the significance of the findings however, as in the 5-HT_{2C} analysis (serine dominant model), 32.14% of responders had a serine allele.

In the non-responder group, 25.49% had a serine allele (Mantel-Haenszel $X^2=0.57$, 1 d.f.; $p=0.45$). Similarly, in the 5-HTTLPR analysis, (short allele dominant model) 67.8% of responders had a short allele, compared with 67.92% of the non-responder group (Mantel Haenszel $X^2=0.00$, 1 d.f.; $p=0.989$). Although the sample is a heterogenous group, all patients fulfilled DSM3R/4 criteria for major depression.

4.5 CONCLUSIONS

In the present study we were unable to confirm the previous findings of a contribution of the 5-HTTLPR polymorphism to patient response when on serotonergic anti-depressant medication. Possible reasons for the failure to replicate are discussed above. It is of note that the previous findings were also not consistent between studies. Although a positive contribution of the 'l' allele has been more consistently seen and better predicted by the prior animal studies, the Korean study (Kim et al, 2000) showed a positive effect of the short allele. This may reflect ethnic diversity as this is the only study to show the 's' version being correlated with response.

This is the first study to consider the role of the 5-HT_{2C} receptor polymorphism in the response of depressed patients to serotonergic medication. Although a meta-analysis appears to show a positive effect of the serine variant in relation to clozapine response in schizophrenia (Sodhi et al, personal communication), this has not been borne out with regard to SSRIs and clomipramine in our study.

A further issue which could not be explored relates to the fact that approximately 30% of patients responding to SSRIs and clomipramine in treatment trials in depression would also respond to placebo. It is therefore unclear in our sample which individuals would be considered as placebo responders. One further way to analyse the data, in an attempt to overcome this difficulty, is to calculate rates of response and non-response in only those individuals who appear to be treatment resistant in spite of numerous complete trials of anti-depressant treatment. This step would require additional clinical information and is therefore not possible at present but such data would provide a clearer picture as to whether analysis of polymorphisms has any predictive value in detecting likely non-responders and treatment resistant patients.

CHAPTER 5

THE EFFECT OF ALLELIC VARIATION OF THE SEROTONIN 2-C RECEPTOR ON FUNCTIONAL RESPONSES TO META- CHLOROPHENYLPIPERAZINE

5.1 BACKGROUND

As described in Chapter 1, one 5-HT_{2C} receptor polymorphism results in a serine substitution in place of cysteine following from a C-G variation at codon 23 (nucleotide 68) (Lappalainen et al, 1995). It is of clinical relevance to know if there is evidence that the two receptors are functionally different. Although initial in vitro work on receptors expressed on xenopus oocytes showed equal concentration/response curves to 5-HT application, later work has shown a difference in binding affinity (Goldman, 1995).

A number of early mammalian studies established a possible role for the 5-HT_{2C} receptor in relation to appetite, prolactin secretion and locomotor activity (Kahn and Wetzler, 1991). In a study of 'knockout mice', those without 5-HT_{2C} receptors became obese and had a lower threshold for seizures induced by audiogenic stimulus (Tecott et al, 1995).

As further detailed in the introduction, in human studies it has been found that the relatively specific 5-HT_{2C} agonist - meta-Chlorophenylpiperazine (m-CPP) causes increases in anxiety, temperature and prolactin levels, together with reduced appetite (Cowen et al, 1995) and decreased slow wave sleep in healthy subjects (Katsuda et al, 1993). In studies involving psychiatric patients m-CPP has been found to exacerbate obsessional symptoms (Hollander et al, 1993), precipitate panic attacks (Charney et al, 1987) and increase formal thought disorder in patients with schizophrenia (Iqbal et al, 1991).

The aim of the present study was to establish whether functional differences exist between two groups of healthy volunteers exhibiting polymorphic variation at the 5-HT_{2C} receptor using previously established neurobehavioural measures of likely 5-HT_{2C} function, including anxiogenic, thermic, hormonal and hypophagic responses to m-CPP effects.

5.2 METHODS

5.2.1 Subjects

Subjects were recruited from the volunteer register maintained in the Psychopharmacology Research Unit (now Neurosciences), Oxford University Department of Psychiatry, as well as from blood donors who were approached when presenting to donate blood, as coordinated by the Oxfordshire Blood Transfusion Service (See Chapter 2).

The included subjects gave signed, informed consent to a buccal swab being taken for genotyping and they also completed a questionnaire requesting demographic details and information regarding past psychiatric history, and history of migraine. No one was taking psychotropic medication. Ethical approval for the study was granted by the local Ethics Committee (OPREC). Subjects were divided into groups by gender and by 5-HT_{2C} polymorphism, either cysteine or serine substituted. Following this initial phase and in view of the clozapine response data in schizophrenia suggesting a serine dominant model correlates more highly with response (Sodhi, 1995), 13 women with a serine substituted allele (2 homozygotic - Ser/Ser, 11 heterozygotic - Cys/Ser), mean age 38.8 years (range 22-60 years), and for comparison, 15 women with homozygosity for the cysteine allele (Cys/Cys), mean age 30.9 years (range 18-56 years), were included in the study (See Table 5.1).

Table 5.1: Female subjects included in the 5-HT_{2C} function study, by subtypes of allelic variation, tabled according to a serine dominant model.

Genotype	Homozygotic	Heterozygotic	Total
Serine	2	11	13
Cysteine	15	0	15

The subjects had no history of psychiatric illness or of significant physical illness which would preclude neuroendocrine testing or complicate interpretation of the results. It had originally been planned to include male subjects in the study however relatively fewer males volunteered to donate blood.

In addition, an insufficient number were found to be serine substituted. In view of this, it was decided to limit the analysis to females in the first instance.

5.2.2 Genotyping

DNA was extracted from the buccal swabs and blood samples using standard methods, as described in Chapter 2 and amplification was achieved using PCR, with primers directed at nucleotides 40-60 and 324-345 of the human 5-HT_{2C} receptor gene. PCR products were analysed by dot blotting using allele-specific - 5'³²P - labelled oligonucleotides. The subjects genotype was confirmed by independent restriction analysis of a 104bp fragment using the Hsp9211 isoschizomer of Nla111, as described (Burnet et al, 1997).

5.2.3 Neuro-endocrine challenge procedure

5.2.3.1 m-CPP dose

The subjects received m-CPP on one occasion and placebo on the other, packaged in matching opaque capsules at a dose of 0.4mg per kg body weight. It was decided to use oral m-CPP, as an extended effect of the drug is reported by comparison with intra-venous administration, as described in the paroxetine study (Chapter 3). This was important in order to ensure that any possible hypophagic effect would not have decreased by the time the test meals were presented to the subjects.

Previous work has established the appropriateness of the model and the dosage schedule needed to reliably reduce appetite under test conditions (Walsh et al, 1994).

5.2.3.2 Sampling protocol

Subjects were tested after an overnight fast at 09.00 hours on two occasions (mean \pm SEM intervals 5.0 ± 1.1 days) in a double-blind, placebo-controlled balanced order, cross-over design. All of the women were tested in the follicular stage of their menstrual cycle, which was established at the first clinical interview. Following an initial 30 minute rest period for base-line blood sampling the m-CPP or placebo was administered. The venous blood samples were taken and oral temperature measured over the next 3 hours at half hourly intervals.

5.2.4 Test meal procedure

Following the neuroendocrine challenge test subjects were given a test meal of pre-selected sandwiches made following a standardized preparation schedule leading to a known approximate calorie content (See Appendices 4 and 5). Preference had previously been established, as described in Chapter 2, and all of the subjects had consumed a test meal in advance of the study to establish whether amounts consumed on the test day were similar to the previous quantity consumed.

The sandwiches were presented to excess with an instruction to consume the quantity desired rather than to eat the maximum number possible. The meal was provided in an isolated setting, with water available to drink, and there was no time limit for the subjects.

5.2.5 Subjective ratings

At each time point following withdrawal of the base-line sample, visual analogue scales were completed for “drowsy”, “anxious”, “nausea”, and “light-headed”, as previously described. These were completed by the choosing of points on 10cm scales with divisions marked at 10mm intervals, as described in Chapter 2. It was thought important to obtain these VAS measures in order to assess whether the quantity of sandwiches eaten was influenced by factors other than the m-CPP administered.

5.2.6 Statistics

Plasma samples were analysed for prolactin, cortisol and m-CPP and calculated, from base-line as area under the curve (AUC), as previously described (Quested et al, 1997; Sargent et al, 1998). For each measure of interest, both physiological or behavioural, values derived on the placebo day were subtracted from those derived on the m-CPP day, in order to yield a net response to the m-CPP challenge. Data were analysed by factorial ANCOVA with genotype as the between-subjects factor and m-CPP levels as the covariate.

Differences on VAS measures between groups were compared as peak change from baseline by Student's t-tests.

5.3 RESULTS

5.3.1 m-CPP drug levels

Plasma m-CPP levels were a potential significant covariate of all the functional responses and adjusted means of the measures were therefore calculated in the comparisons of the two groups.

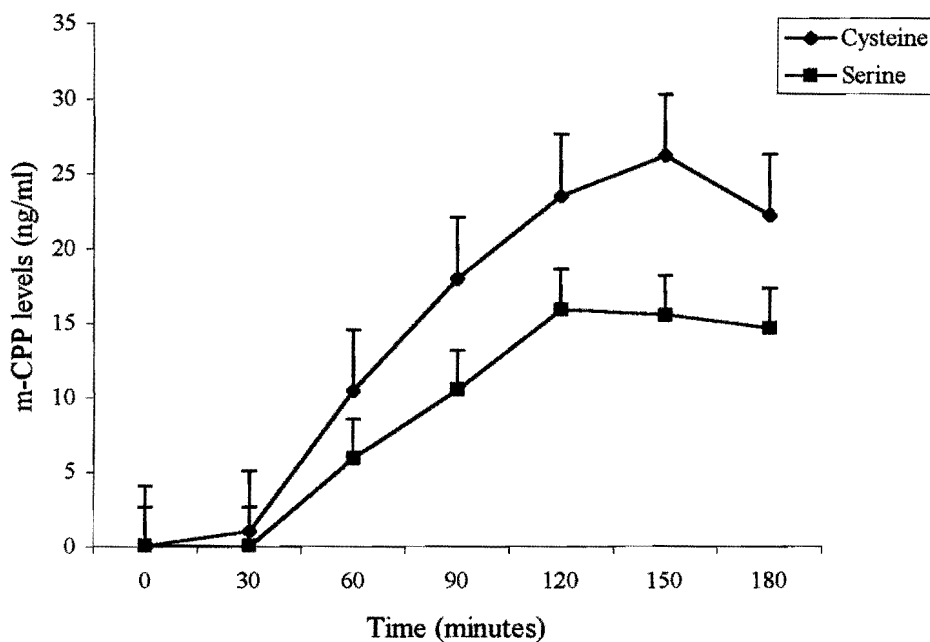


Figure 5.1: Comparison of m-CPP drug levels in ng/ml (mean±SEM) in two groups differing by allelic variation at the 5-HT_{2C} receptor. The levels did not differ significantly by comparison of AUC measures, in ng/ml x minutes (P=0.16, Student's t-test).

The techniques used in the analysis of the serum levels have been discussed in detail in Chapter 2. Mean drug levels for the two groups can be seen in Figure 5.1.

5.3.2 Effect on food intake

None of the responses varied significantly with genotype when analyses were carried out after covariation with m-CPP levels, however the hypophagic effects of m-CPP were significantly attenuated in subjects with a 5HT_{2C} Ser receptor allele prior to covariation with m-CPP levels ($p < 0.05$) to the extent that no reduction is seen and the quantities of sandwiches consumed on the m-CPP and placebo day are similar (See Table 5.2).

Table 5.2: Difference in quantity of sandwiches consumed between the two test days, on m-CPP or placebo, testing for the m-CPP induced hypophagic effect. Each sandwich is a quarter of a round (one round is 2 slices of medium thickness bread). Result is significant (Student's t-test; $p = 0.017$) prior to covariation for m-CPP levels.

Genotype	Mean sandwich intake (SEM)		Difference (SEM)
	m-CPP day	Placebo day	m-CPP – Placebo
Cysteine	4.53 (0.64)	6.07 (0.53)	-1.54 (0.54)
Serine	5.85 (0.77)	5.46 (0.56)	0.39 (0.51)

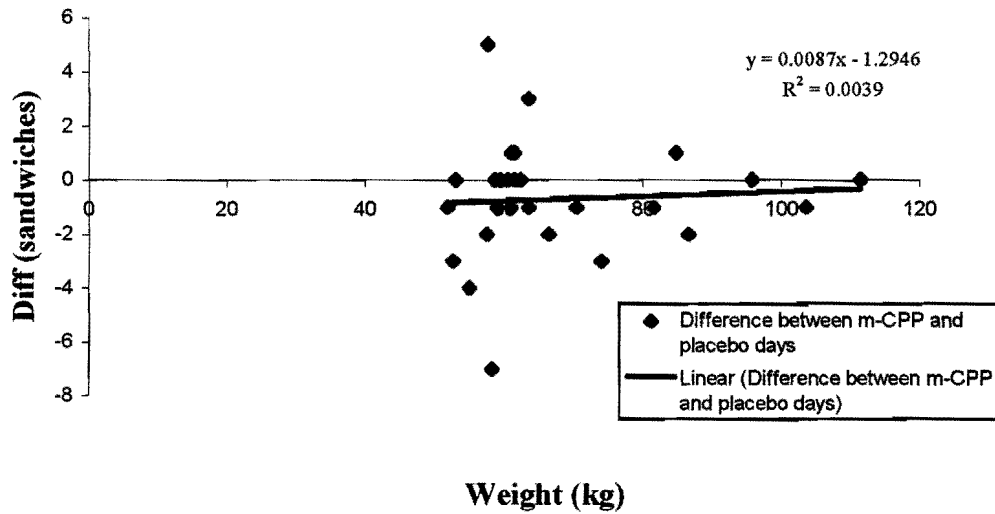


Figure 5.2: Correlation of body mass in kilogrammes with difference in sandwich intake between m-CPP and placebo days ($p > 0.05$ - $R^2 = 0.0039$).

There was also no significant effect seen when calories were used as the measure of possible difference in appetite. Although the sandwich preparation was standardized in order to ensure that each sandwich of a particular type had a similar calorific content to other sandwiches of the same type, there were absolute differences in calorie content between the types (See Table 5.3).

Table 5.3: Difference in calorie intake between m-CPP and placebo days, after covarying for m-CPP levels. Adjusted means are shown (Student's t-test; $p = 0.14$).

Genotype	Change in mean calorie intake (\pm SEM)
Cysteine	-87 ± 37
Serine	-2 ± 41

The differences between the m-CPP and placebo days were correlated with body weight to assess whether this could have caused a systematic effect on the direction of the results.

As the mean body mass of the subjects differed between the two groups defined by possession of the 5-HT_{2C} allele, it had to be established whether this general difference between the two groups could be biasing the measures of outcome related to diet and food intake. This was not found to be the case as the coefficient of correlation was not significant (See Figure 5.2; $p > 0.05$).

5.3.3 Prolactin and cortisol levels

Prolactin levels were measured using the techniques described in detail in Chapter 2.

The data show that this particular 5-HT_{2C} polymorphism does not seem to influence endocrine parameters following m-CPP.

Data regarding the difference between the two groups by polymorphism, according to a serine dominant model, is presented in Table 5.4 and the variation in prolactin levels between m-CPP and placebo days for each group is shown in Figures 5.3 and 5.4.

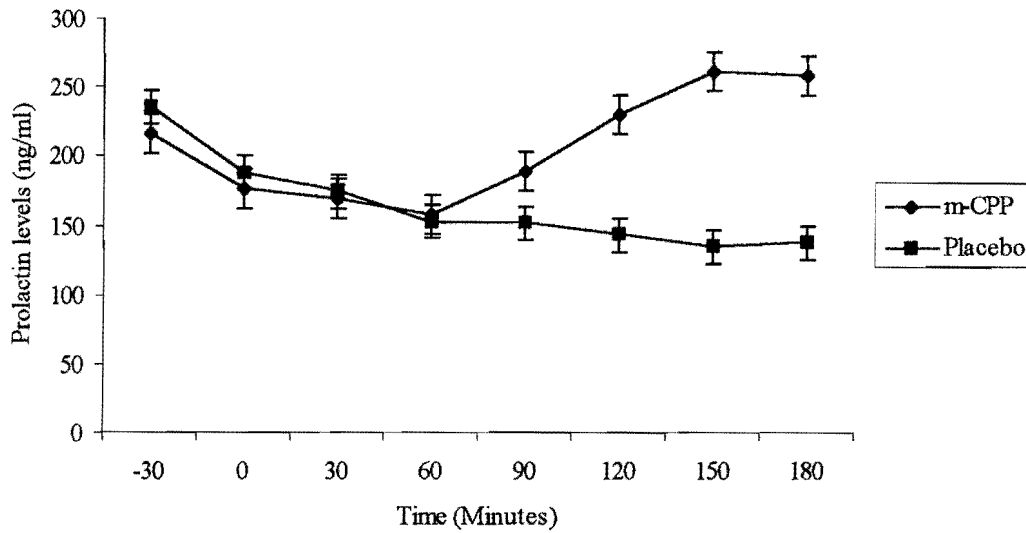


Figure 5.4: Prolactin levels in ng/ml (mean±SEM) in serine subjects. Comparison of AUC measures (ng/ml x minutes) between m-CPP and Placebo days (change from baseline) is significant ($p < 0.05$, Student's t-test).

Although there is a stimulatory effect on prolactin by m-CPP, as was discussed in Chapters 1 and 3, there is no significant effect of the polymorphism. This is also the case with cortisol where increases are seen on the m-CPP days (Figure 5.5) but not between the groups according to the polymorphic variation (Table 5.5).

Table 5.5: Mean difference in cortisol levels between m-CPP and placebo days according to change from baseline AUC measures by gene prior to covariation with m-CPP levels. (Comparison of means by Student's t-test: $p = 0.565$).

Genotype	Mean cortisol level (\pm SEM) ($\mu\text{g}/100\text{ml} \times \text{minutes}$)
Cysteine	946 ± 336.18
Serine	662 ± 283.6

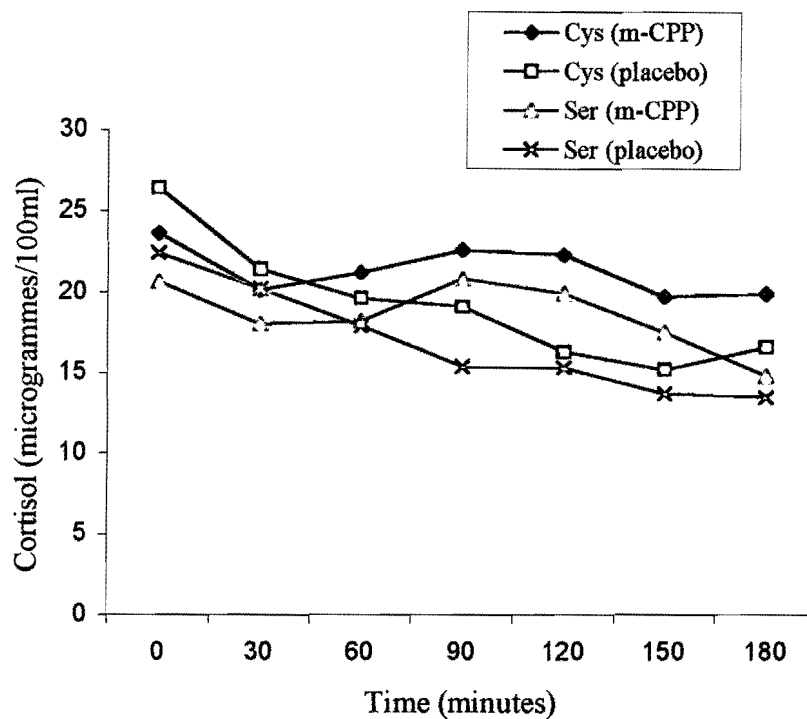


Figure 5.5: Cortisol levels (microgrammes/100ml) in all subjects. Comparison between m-CPP and placebo days in serine and cysteine substituted groups. Error bars are omitted for clarity. No significant group difference on factorial ANCOVA with m-CPP as a covariate using adjusted means ($p=0.99$).

5.3.4 Thermic responses

The data shows that the polymorphism does not seem to influence thermic parameters following m-CPP significantly although it is apparent from inspection of the AUC measures (See Figure 5.6) that the cysteine group exhibits almost no effect on temperature until the final two time points whereas the serine group appears to show a more marked positive effect over time. This result is therefore in contrast to the hypophagic effect although following covariation for m-CPP levels the result similarly remains non-significant.

Table 5.6: Comparison of difference in temperature levels between m-CPP and placebo days by gene following covariation for m-CPP levels. Adjusted AUC means are shown (Factorial ANCOVA; $p=0.50$).

Genotype	Mean temperature diff. (\pm SEM) ($^{\circ}\text{C} \times \text{minutes}$)
Cysteine	5.5 ± 5.2
Serine	0.11 ± 5.6

When the group was considered as a whole, the expected positive thermic effect of m-CPP was seen, confirming that there was no other explanation for the failure to establish a difference between the two groups with different 5-HT_{2C} alleles. As can be seen in Figure 5.6, the m-CPP day temperatures are higher than placebo day temperatures when shown as change from baseline.

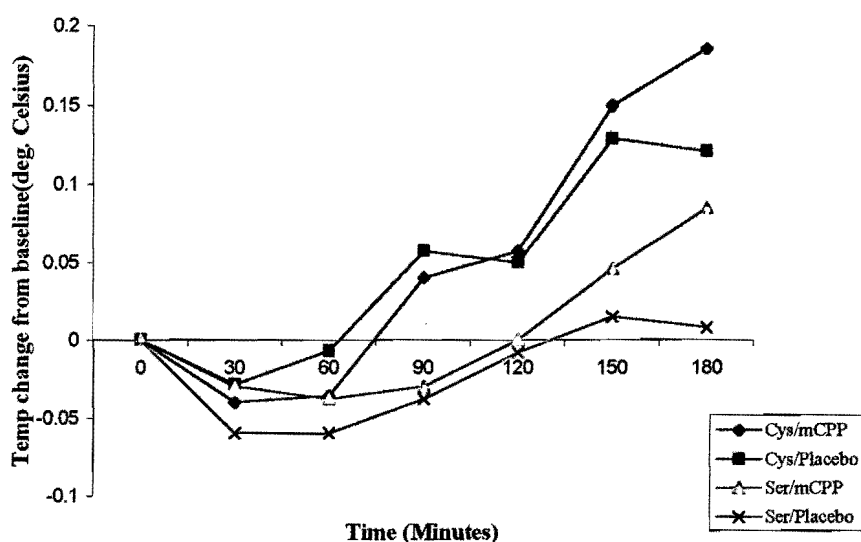


Figure 5.6: Comparison of temperature measurements ($^{\circ}\text{C}$) between the two groups differing according to possession of a Cys or Ser polymorphism, depicted as change from baseline (N.S – see table 5.6). Error bars are not shown for clarity.

5.3.5 Visual analogue scores

While the differences between m-CPP challenge days and placebo days were predictable due to direct effects of the drug, as seen in other m-CPP studies, it was not known whether the two groups would exhibit differences as an effect of the polymorphic variation. As discussed in Chapter 1 and shown in Chapter 3, m-CPP has been demonstrated to enhance anxiety in normals and in those predisposed to anxiety as a disorder (Charney et al, 1987). Measures differing between the groups at a statistically significant level prior to covarying for m-CPP levels include 'anxiety' and 'light-headedness'. For all figures shown, occasion 1 is the 'm-CPP' day and occasion 2 is 'placebo' although in practice the order was randomized.

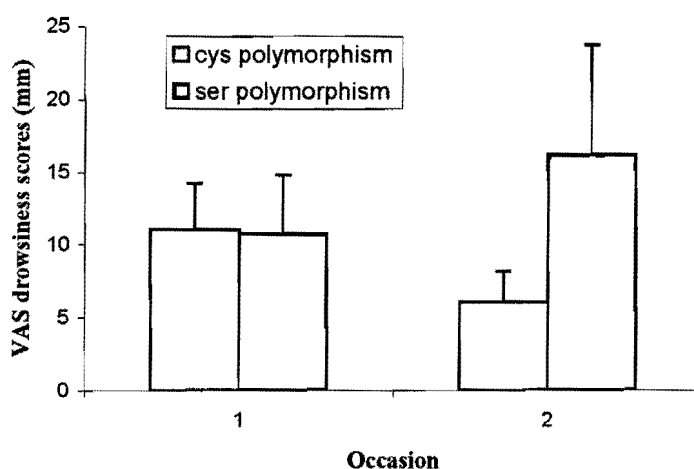


Figure 5.7: Comparison of VAS drowsiness scores in mm (Mean \pm SEM), according to groups differing by polymorphism (cysteine and serine) on m-CPP and placebo days (Student's t-test; $p=0.318$). Occasion 1 is m-CPP.

For ‘drowsiness’ (cysteine group only) and ‘nausea’ (both cysteine and serine groups) measures on the VAS scales, an increase was seen on the m-CPP days compared to the placebo days (See Figures 5.7 and 5.10) however Student’s t-test of the difference between m-CPP and placebo days according to the two groups by 5-HT_{2C} polymorphism is non significant (p=0.318 and 0.715 respectively).

Anxiety was found to be highest in the cysteine group on the m-CPP day (See Figure 5.8). This was not significant in comparison with the serine group after subtraction of the placebo day from the m-CPP day and comparison of the difference between group means measured for the peak change from baseline by Student’s t-test (p= 0.197).

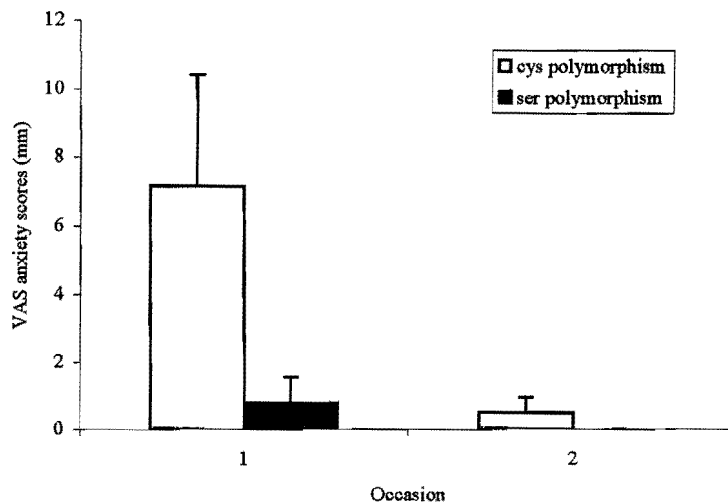


Figure 5.8: Comparison of VAS anxiety scores in mm (Mean±SEM) according to group differing by polymorphism (cysteine and serine) on m-CPP and placebo days (Student’s t-test; NS; p=0.197). Occasion 1 is m-CPP and occasion 2 is placebo.

Light-headedness measures were not found to be significantly different between the two groups, with probability at the $p=0.332$ level, by Student's t-test (See Figure 5.9). Understandably, the m-CPP day was seen to be worse for both groups on this measure but to a much greater degree on the m-CPP day for the cysteine group.

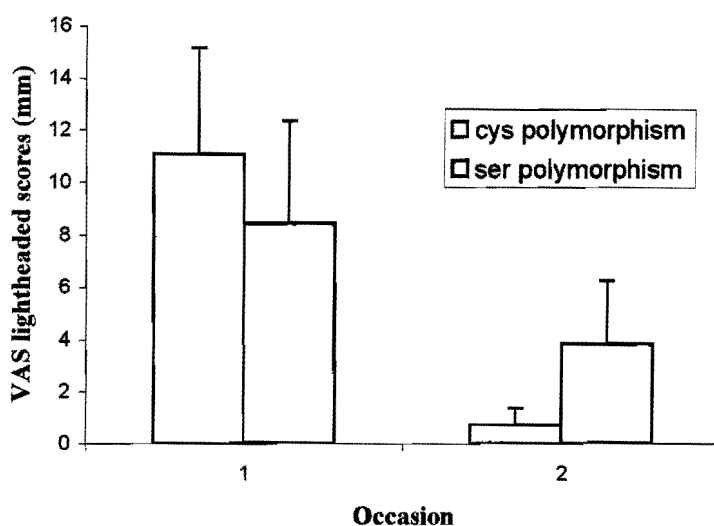


Figure 5.9: Light headedness VAS measures in mm (Mean \pm SEM) compared between groups differing by the two polymorphisms. The difference is not significant between the two groups ($p=0.332$; Student's t-test). Occasion 1 is m-CPP and occasion 2 is placebo.

The difference between the groups is again not significant for the VAS score concerning light-headedness. For the nausea measure, although there is a clearly visible difference between the m-CPP and placebo days, there is no statistical difference between the two groups by genotype (See Figure 5.10).

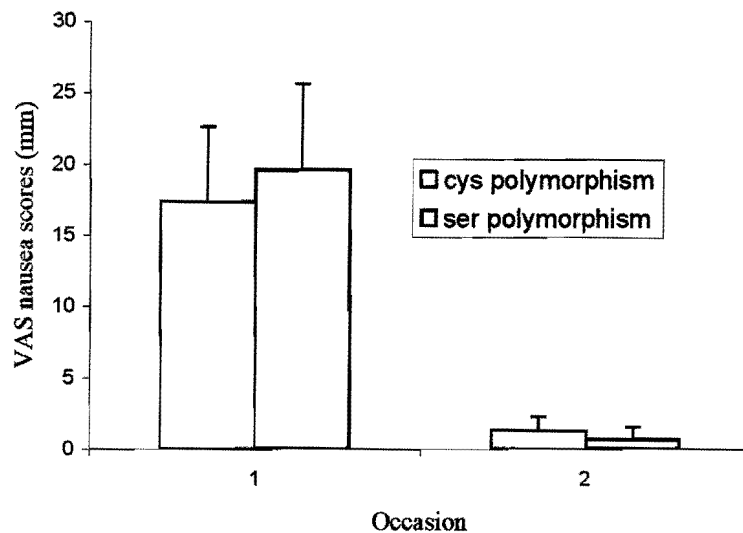


Figure 5.10: Nausea VAS scores in mm (Mean±SEM) compared between groups differing according to the two polymorphisms. Difference between m-CPP and placebo days and between groups is non-significant ($p=0.715$ by Student's t-test). Occasion 1 is m-CPP and occasion 2 is placebo.

5.4 DISCUSSION

The predicted difference of the hypophagic effect of m-CPP was seen with an enhanced effect in the cysteine group, compared to the serine group but, although the expected reduction of calorie intake was seen in the whole sample ($p=0.045$) only a non-significant trend was seen between the two genotyped groups (See Table 5.3).

There was also no variation between the groups in the effect of m-CPP on prolactin and cortisol levels. An increased temperature was seen on the m-CPP day in both groups but no significant difference was seen between the two groups.

In view of the binding and the clozapine response data it was predicted that functional differences would be found between the genetically differing groups. As the results show a trend in the predicted direction for appetite (ie: a greater hypophagic effect in cysteine patients) it may be that a larger sample is required, or more sensitive measures. Previous work has demonstrated a negative effect of m-CPP on appetite using a full buffet meal, possibly a more sensitive measure of appetite than that used in the present study (Walsh et al, 1994).

It is unlikely that the dose of m-CPP was too small as a reduction of calorie intake was seen across the whole sample on the m-CPP occasion and blood levels of m-CPP were as expected. In view of X-chromosomal inactivation in females (Migeon, 1994), it is possible that the responses of the heterozygote females may have been equivalent to the cysteine substituted rather than the serine substituted homozygotes, which would have confounded the outcome.

5.5 CONCLUSIONS

As discussed above the present data set does not support a significant functional effect of this 5-HT_{2C} polymorphism on endocrine, behavioural or thermic parameters.

The most interesting finding is that of a trend towards significance in the reduction of food intake following m-CPP in those individuals with a cysteine allele. This finding was significant prior to covarying for m-CPP levels, a necessary step in establishing a direct link between the 5-HT_{2C} agonist and a measurable functional effect.

Subjects with serine substituted receptors, 5-HT_{2C} Ser, consumed approximately 80 calories more on the day of m-CPP administration than those with cysteine genotype, 5-HT_{2C} Cys. While the difference in hypophagic effect is not currently significant once m-CPP levels have been covaried for, it is evident that a sample size of twice the current number would be needed for 80% power to exclude the effect of the genotype, assuming a functional response of 25% to be of likely clinical significance. In order to establish whether there is a recessive effect of the allele it would also be necessary to include a sufficiently large group of 5-HT_{2C} Ser receptor homozygotes. In view of reported gender differences in measures of response to m-CPP it would also be important to carry out similar investigations in male subjects (Bagdy and Arato, 1998).

CHAPTER 6

THE EFFECT OF ALLELIC VARIATION IN THE SEROTONIN TRANSPORTER PROMOTER ON FUNCTIONAL RESPONSES TO CLOMIPRAMINE

6.1 BACKGROUND

The serotonin transporter (SERT) has been increasingly studied in view of its role in the sodium dependent pre-synaptic re-uptake of serotonin during neurotransmission in the serotonergic system (Rudnick and Clark, 1993). It is the target of numerous antidepressant drugs, as both the tricyclic antidepressants and the SSRIs occupy sites overlapping the 5-HTT binding site (Schloss and Betz, 1995). As discussed in Chapter 1, the 5-HTT derives from a number of different polymorphic variants.

In this study intravenous clomipramine was administered to normal subjects in order to establish whether there may be a functional difference relating to allelic variation in the serotonin transporter promoter-polymorphism (5-HTTLPR), which occurs in long (l) and short (s) forms (As discussed in Chapter 1).

The 'l' form has been associated with greater expression of 5-HTT sites (Lesch et al, 1996; Little et al, 1998) and it is therefore important to evaluate whether human brain function might be influenced according to the possession of a specific promoter polymorphism.

As previously described, it is possible to assess human 5-HT function in the brain using a neuro-endocrine paradigm, by measuring fluctuations in hormones secreted by the anterior pituitary gland as a proxy marker for central neurotransmitter activity (Cowen, 1993). In this study prolactin levels were monitored as a marker of the increase in 5-HT neurotransmission which occurs with acute 5-HT reuptake blockade.

6.2 METHODS

6.2.1 Subjects

Subjects were either recruited from the volunteer database in the Psychopharmacology Research Unit or at local blood donor clinics. Following the screening of volunteers at the blood donor clinics in Oxford, two samples of seven female homozygote subjects each were identified from those without a history of psychological problems, possessing 'll' or 'ss' genotypes. The subjects were interviewed using the structured interview for DSM IV (SCID). It was thereby determined that no subjects had a current or previous history of an axis-one psychiatric diagnosis in DSMIV.

The mean age of the women with the 'll' genotype was 40 years (range 23-60 years) while that of the 'ss' subjects was 38 years (range 22-54 years). One woman in each group was post-menopausal. All tests were carried out in the follicular stage of the menstrual cycle, where appropriate, as previously discussed in Chapter 1. The mean body weight of the 'll' subjects was 72.5 kg (range 60.8-94.8 kg) and that of the 'ss' subjects was 67.7 kg (range 55-87.6 kg). Subjects gave signed consent to the study which had been approved by the local Ethics Committee, OPREC.

6.2.2 Genotyping

DNA was extracted from cheek swabs provided by the blood donor volunteers or from blood taken by venous samples from other volunteers to the Psychopharmacology Research Unit, following their informed and signed consent. Genotyping of the polymorphism was carried out as previously described (Lesch et al, 1996).

The oligonucleotides were directed against bases -1416 to -1397 and -910 to -888 of the human SERT gene, giving rise to 484 ('s' allele) or 528 ('l' allele) basepair fragments. PCR was carried out in a final volume of 20 microlitres, with 12% DMSO, using cycling conditions as described in Chapter 2 (Klauck et al, 1997). Products were then resolved on a 3% agarose gel by electrophoresis with ethidium bromide and DNA molecular weight markers.

6.2.3 Neuroendocrine protocol

Subjects were tested at 09.00 hours after an over-night fast in a single-blind, balanced-order, placebo-controlled design. Following insertion of an in-dwelling venous cannula, a 30 minute rest period elapsed after which clomipramine or placebo (saline) was infused intravenously over 15 minutes.

The dose of clomipramine was calculated as 0.1 mg per kg on the basis of previous studies showing that this dose of clomipramine produced a reasonably well tolerated increase in plasma prolactin and the sample size was also selected in view of a measureable effect being achieved with clomipramine at significant levels in previous studies (McCance et al, 1989). Venous samples for prolactin and clomipramine were taken from the cannula, following the initial removal of 10ml of blood for discarding on each occasion, at 15 minute intervals for the next 120 minutes. The mean interval between the tests was 25 days (range 2-134 days) for the 'll' group and 14 days (range 5-56 days) for the 'ss' group.

6.2.4 Subjective measures

Subjects rated themselves for 'happiness', 'anxiety', 'drowsiness', 'dizziness' and 'nausea', every 30 minutes using a 10cm visual analogue scale, as previously described in Chapter 2.

6.2.5 Biochemical assays

Plasma prolactin concentration was measured by standard immunoradiometric assay and clomipramine levels were determined by high performance liquid chromatography with ultra violet detection (Anderson et al, 1992), as described in Chapter 2.

6.2.6 Statistics

A two-way repeated measures analysis of variance (ANOVA) was used for the analysis of prolactin and cortisol levels with 'treatment' (clomipramine vs saline) and 'time' as within-subject factors while 'genotype' (ll vs ss) was the between-subjects factor. The Huynh-Feldt correction was applied as is always done where the assumption of sphericity is violated. In addition to the use of the above method, prolactin levels were also calculated as an AUC measure by the trapezoid method following the subtraction of baseline values at time '0'. To derive a 'change in AUC' measure (Δ AUC), placebo day values of prolactin were then subtracted from the responses following clomipramine administration.

Clomipramine levels were also calculated according to the above AUC method. VAS ratings were calculated as peak increase over base-line and the placebo day value was subtracted from the clomipramine test day to give an eg: 'change in nausea' measure (Δ nausea).

6.3 RESULTS

6.3.1 Endocrine Measures

Administration of the intravenous clomipramine produced a significant increase in plasma prolactin relative to placebo saline infusion as shown by a significant treatment by time interaction on a repeated measures analysis of variance (ANOVA) ($F=4.48$, $P<0.001$).

See Figure 6.1, below.

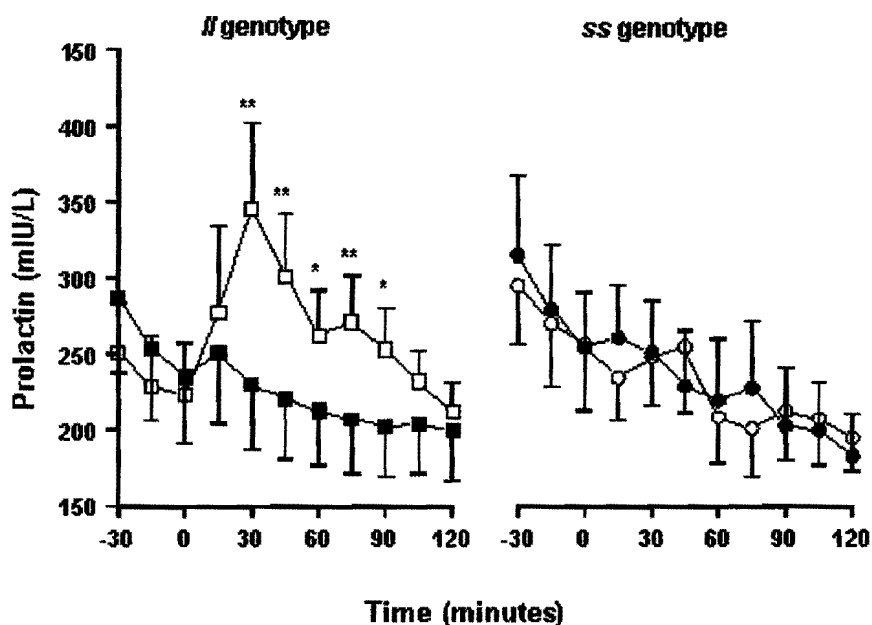


Figure 6.1: Plasma prolactin levels (mean \pm SEM) in two groups of female volunteers before and after clomipramine (open symbols) and saline (closed symbols). Subjects with an 'll' genotype had a significantly greater response to clomipramine than saline.

(* $p<0.05$; ** $p<0.01$; Fisher's test of least significant difference)

There was no main effect of genotype ($F=0.024$, $P=0.88$), but a significant occasion by time by genotype interaction ($F=3.32$, $P=0.005$). In comparison with saline, clomipramine significantly increased plasma prolactin in subjects with the 'll' genotype but not in those with the 'ss' genotype (See Figure 6.1). However, there were no baseline (mean \pm SEM) differences between prolactin levels in the 'll' and 'ss' groups (256 \pm 43 vs 224 \pm 34 mIU/l respectively, $P=0.56$, unpaired t-test).

6.3.2 Subjective Measures

The increase in subjective nausea (change from baseline) produced by clomipramine did not differ between the genotypes (27.0 \pm 11.5 vs 27.4 \pm 9.0 mm, $P=0.98$, unpaired t-test) (See Figure 6.2). The other measures are summarised in Figures 6.3 to 6.6.

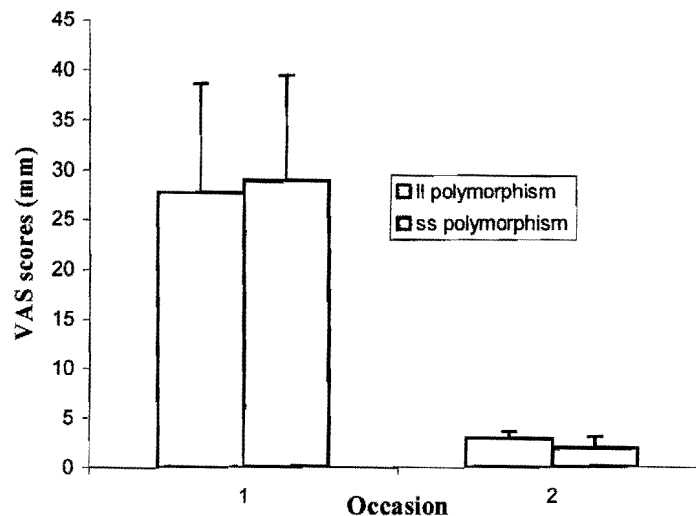


Figure 6.2: Nausea scores measured on Visual Analogue Scales (mm). Occasion 1 is clomipramine day and occasion 2 is placebo day. No significant difference is seen on the measure (placebo subtracted or Δ nausea) between genotypes 'll' and 'ss' ($P=0.98$, paired Student's t-test).

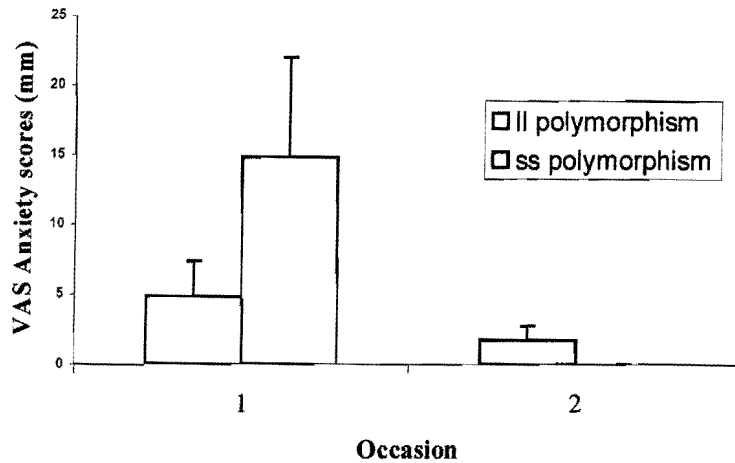


Figure 6.3: VAS scores for anxiety compared between the two variant groups. Occasion 1 is clomipramine and occasion 2 is placebo. Difference is not statistically significant on the measure (placebo subtracted or Δ anxiety) between the 'll' and 'ss' groups. ($p=0.186$ by paired Student's t-test).

While the chart concerning the VAS anxiety (Figure 6.3) scores suggests that females with the 's/s' genotype experience higher anxiety levels on the clomipramine day, the statistical significance is at the trend level rather than at $p<0.05$.

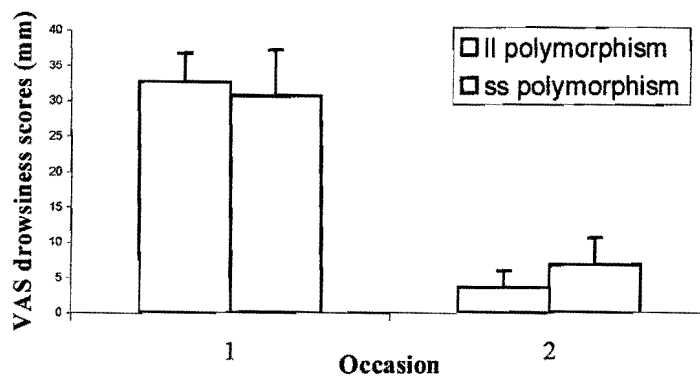


Figure 6.4: VAS scores for drowsiness compared between the two variant groups. Occasion 1 is clomipramine and occasion 2 is placebo. Difference is not statistically significant on the measure (placebo subtracted or Δ drowsiness) between the 'll' and 'ss' groups. ($p=0.719$ by paired Student's t-test).

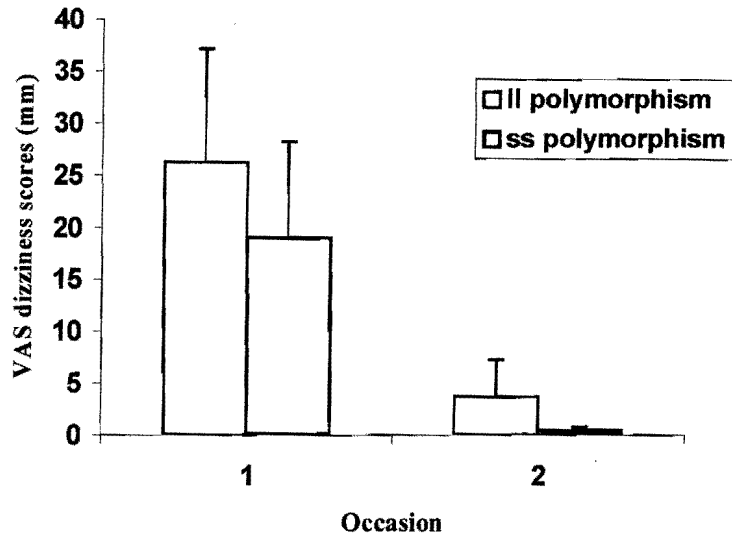


Figure 6.5: VAS scores for dizziness compared between the two variant groups. Occasion 1 is clomipramine and occasion 2 is placebo. Difference is not statistically significant on the measure (placebo subtracted or Δ dizziness) between the 'll' and 'ss' groups. ($p=0.830$ by paired Student's t-test).

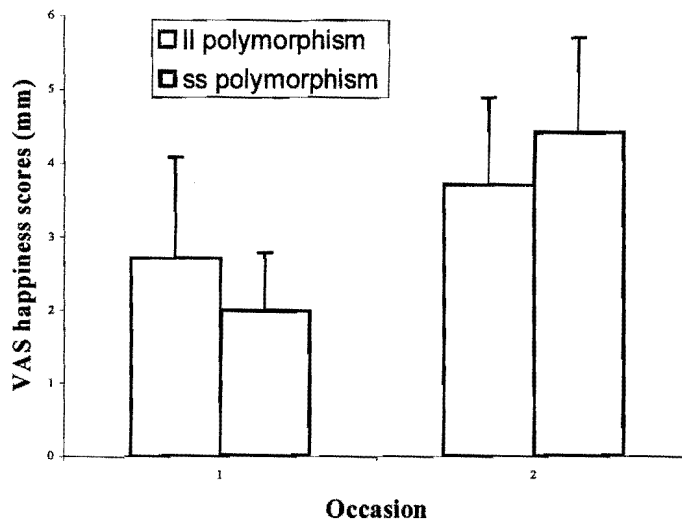


Figure 6.6: VAS scores for happiness compared between the two variant groups. Occasion 1 is clomipramine and occasion 2 is placebo. Difference is not statistically significant on the measure (placebo subtracted or Δ happiness) between the 'll' and 'ss' groups. ($p=0.719$ by paired Student's t-Test).

6.3.3 Clomipramine levels

The mean area under the curve (AUC) of plasma clomipramine following infusion was found to be higher in 'll' subjects in comparison to the 'ss' group, (See Figure 6.7) but not at a statistically significant level.

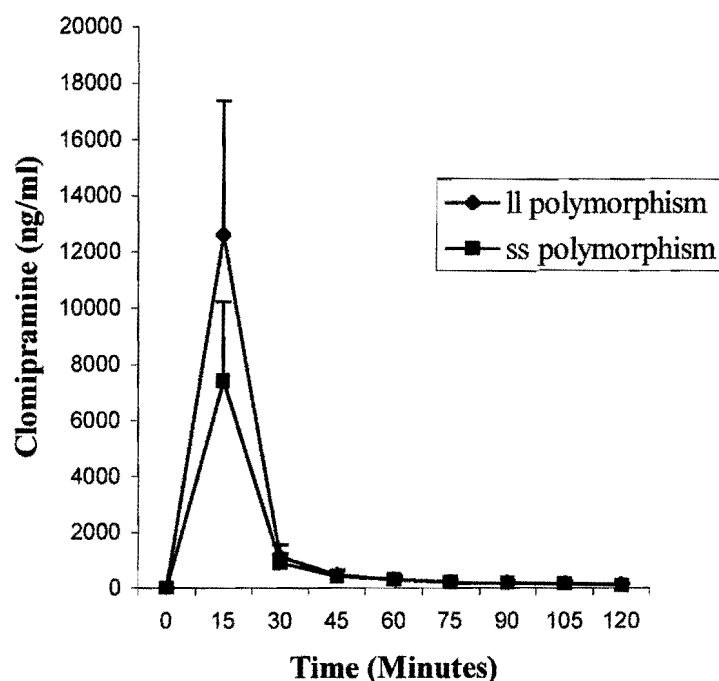


Figure 6.7: Comparison of serum clomipramine levels as measured by High Performance Liquid Chromatography. Actual levels in mIU/ml are displayed however the statistical analysis was carried out using AUC measures (Student's t-test; $p > 0.05$)

To control for the possible pharmacokinetic effects of different plasma clomipramine levels, the AUC clomipramine levels were co-varied with the prolactin response to clomipramine (change in prolactin AUC) in a factorial ANCOVA.

This showed no significant effect of clomipramine level on prolactin response ($F=2.89$, $P=0.12$) but the prolactin AUC was significantly higher in the 'll' subjects (mean \pm SEM Δ AUC prolactin response to clomipramine in 'll' subjects, 126 ± 48 vs -7.2 ± 40 mIUx h/l, $F=7.78$, $P=0.018$).

There were no significant correlations between change in prolactin AUC and the following variables: clomipramine AUC level ($R=-0.14$, $P=0.64$), change in nausea ($R=-0.18$, $P=0.54$), body weight ($R=0.10$, $P=0.73$), or interval between clomipramine and placebo tests ($R= -0.28$, $P=0.33$).

6.4 DISCUSSION

The results as discussed above suggest that subjects with the 'll' genotype have an enhanced prolactin response to clomipramine. In the case of the subjects with the 'ss' genotype little response was seen. This would suggest that the 'll' genotype is associated with increased 5-HT response to 5-HT re-uptake blockade, presumably at 5-HT uptake sites in nerve terminals in the hypothalamus, although it is acknowledged that the sample size in this study is somewhat small. Other studies which may have relevance to this finding include a report suggesting that depressed patients with the 'ss' genotype may respond less well to the SSRI fluvoxamine than heterozygotes with at least one 'l' allele (Smeraldi et al, 1998). In the latter study administration of pindolol (a 5-HT_{1A} receptor antagonist) improved the response rate so that the 'ss' homozygotes' responses looked more similar to the 'ls' and 'll' groups.

As 5-HT_{1A} receptor antagonists promote an increase in 5-HT neurotransmission it is possible that the 'ss' genotype in this study failed to show a corresponding increase similar to the 'll' group in view of excessive activation of cell body 5-HT_{1A} auto-receptors which are inhibitory to 5-HT cell firing (Adell and Artigas, 1991; Bel and Artigas, 1992).

It would therefore seem in the pindolol study that receptor blockade was preventing extracellular serotonin from causing negative feedback on 5-HT cell-firing, thereby enhancing terminal 5-HT neurotransmission. An alternative explanation for the present finding is that animal studies have shown an increased uptake of serotonin into both lymphoblasts and platelets in association with the 'll' genotype, which suggests an increased expression of transporter sites (Lesch et al, 1996; Collier et al, 1996a; Greenberg et al, 1999). This could lead to an enhanced response to clomipramine with increased prolactin release, should a greater number of transporter sites allow a 5-HT reuptake-blocker to correspondingly enhance 5-HT neurotransmission. Although the above findings could suggest a reduced effect of 'ss' genotypes, this would not correspond well to the animal and experimental studies.

6.5 CONCLUSIONS

Although this is a striking finding with possible relevance for antidepressant treatment response, the effect needs to be replicated and extended to male subjects. It would also be appropriate to test those individuals who are heterozygous for the 'l' and 's' alleles.

It would be of interest for similar studies to be carried out looking at the response to clomipramine challenge in depressed patients. Here many other factors, such as cortisol hypersecretion, which may have a role in the aetiology of the depressive condition, may further complicate response to the challenge and would therefore need to be evaluated accordingly. Some studies have suggested that the cortisol responses to clomipramine might be regulated differently. For example, in one study which investigated the effect of short term treatment with lithium on prolactin and cortisol responses to clomipramine, only the prolactin response was elevated (McCance et al, 1989). One possibility for these kinds of differences is that 5-HT has the ability to increase cortisol secretion directly from the adrenal gland where different 5-HT release mechanisms may be involved.

A possible effect of stress can also not be excluded but the clomipramine was tolerated relatively well and a low dose was chosen for this reason. Clomipramine is highly serotonergic (Attar-levy, 1999), but not particularly selective for the 5-HTT and it would also be preferable to repeat the study with a more highly selective serotonin reuptake inhibitor such as citalopram, as a more direct test of the hypothesis.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

In this thesis a number of specific hypotheses have been tested, within a more general framework linking dysfunction of the serotonin neurotransmitter system to major psychiatric illnesses and, for the purposes of this thesis, specifically to depression.

The studies in this thesis have therefore followed two main lines of investigation :-

- 1) the effect of SSRI treatment on the function of the 5-HT_{2C} receptor and the possible influence of one 5-HT_{2C} polymorphism - cys23ser - on previously established functional indices of 5-HT_{2C} receptor function and, in a similar manner, the influence of variation in the promoter polymorphism of the serotonin transporter on hormonal fluctuation as a proxy measure of functionality.
- 2) the possible relevance of polymorphic variation in the 5-HT_{2C} receptor (cys23ser) and in the promoter polymorphism of the serotonin transporter, to treatment response, with the use of SSRIs in depression.

In the first experimental chapter (Chapter 3). it was established that a three week period of treatment on the SSRI paroxetine was followed by significant attenuation of both the prolactin and hyperthermic responses to intra-venous m-CPP challenge, a relatively selective 5-HT_{2C} agonist, in healthy subjects, as predicted by the theoretical model derived from previous studies concerning patients suffering from OCD and animal studies.

The study differed from previous work in the field as m-CPP was administered intravenously, in order to avoid the difficulties relating to variation in plasma drug levels by the effect of anti-depressant drugs on hepatic metabolism. Possible causes of a finding at odds with the original hypothesis were thought to be unlikely. These included :-

a) the non-selectivity of m-CPP, with a possibility that 5-HT_{1A} desensitisation could contribute to the reduction in PRL release (Sargent et al, 1997) however this explanation is countered by the fact that body temperature would be expected to fall rather than rise if 5-HT_{1A} receptors were involved (Anderson et al, 1990).

b) a possible argument linking m-CPP - induced PRL release to pre-synaptic 5-HT release stimulating post-synaptic 5-HT_{2C} receptors by an indirect effect. In this scenario SSRIs could block the ability of m-CPP to release 5-HT in a similar manner to how they block the 5-HT releasing effect of d-fenfluramine. This is supported by animal work where SSRIs attenuate m-CPP stimulated PRL release (Baumann et al, 1993). However,

this possibility is countered by the fact that 5-HT neuro-toxic lesions in rats produce an increase in the prolactin response to m-CPP.

This in turn suggests that the prolactin response is through post-synaptic receptors, which in this case are showing an up-regulation (Quattrone et al, 1981).

c) a possible direct antagonist effect of paroxetine was also considered but the affinity of paroxetine for 5-HT_{2C} receptors is known to be considerably less than fluoxetine (Wong et al, 1991) and this explanation is therefore unlikely.

The corollaries derived from the above predictive framework have been supported by more recent work which has upheld the perspective argued in the points listed above, namely that 5-HT_{2C} desensitisation is the most tenable explanation for the data in the paroxetine study (Chapter 3). A 5-HT_{2C} antagonist – SB-242084 – was able to reverse the 5-HT_{2C} mediated anxiogenic effects of fluoxetine, sertraline and m-CPP while the 5-HT_{1A} antagonist, WAY-100635, was not, in a study concerning Sprague Dawley rats (Bagdy et al, 2001). The rate of desensitisation may have relevance to speed of onset of action.

In the second experimental chapter (Chapter 4). we failed to replicate either the three previous studies showing an association between possession of the long allele of the promoter region polymorphism of the serotonin transporter and response to anti-depressants or the one study linking the shortest polymorphism (Kim et al, 2000) with response. In a similar manner, we did not find any any correlation between possession of

a polymorphic variant of the 5-HT_{2C} receptor (serine substitution at codon 23) and the response of patients to SSRIs or clomipramine.

It had been predicted that the polymorphism might be relevant to SSRI response as binding differences between the two forms had been reported and a study had also linked the serine substituted receptor to therapeutic response in schizophrenic patients treated with clozapine (a 5-HT_{2C} receptor blocker), as discussed in Chapter Four (Sodhi et al, 1995).

It is also known that m-CPP aggravates formal thought disorder in schizophrenia and that clozapine has been shown to be particularly effective in patients with the disorganisation syndrome, one of the major features of which is formal thought disorder. A number of lines of reasoning had therefore suggested that the 5-HT_{2C} receptor and its polymorphisms might have some relevance to the treatment of different psychiatric conditions but, in view of the varying nature of the effects on the receptors (agonism vs antagonism), by differing means. Although subsequent work has supported a positive correlation of clozapine response and this serine substitution in schizophrenia, leading to its incorporation in an algorithm with other polymorphisms (Arranz et al, 2000), some studies have not replicated the finding (Masellis et al, 1995; Segman et al, 1997).

In spite of the non-replication of the above finding in some groups, the link between 5-HT_{2C} blockade occurring due to the atypical anti-psychotic drugs and weight gain is well supported by both the knockout mice studies (Tecott et al, 1995), where obesity ensued

and in the clinical studies which have monitored weight gain in patients commencing atypical anti-psychotic drugs (Fenton, 2000).

Although the drugs differ by the degree to which weight is increased, with clozapine and olanzapine contributing to the greatest average gain, there is an overall positive effect on appetite and weight in both the typical and atypical groups, as reported in meta-analyses (Allison et al, 1999).

Alongside the reports of significant weight gain, there are now large scale studies noting a positive association of the atypical drugs with Type 2 diabetes mellitus (Desai et al, 2002), even in the absence of significant weight gain, and an increase in serum leptin levels (Herran et al, 2001).

In an attempt to establish which 5-HT_{2C} polymorphism/s are most highly correlated with obesity, studies have initially examined a possible link between the cys23ser polymorphism and disorders of body mass but association and linkage studies were negative in one study which involved children, adolescents and adults who were obese, normal weight or underweight (Lentes et al, 1997). This finding is analogous to the absence of correlation with either 5-HT_{2C} polymorphism (Cys or Ser) and absolute body mass reported in Chapter Five, which also only reported a trend towards a decreased m-CPP induced hypophagic effect in females with serine substituted 5-HT_{2C} receptors.

In view of the absence of a clear correlation between the cys23ser receptor polymorphism and weight, a further study investigated the possible link between 5-HT_{2C} polymorphisms deriving from the promoter region, obesity and diabetes (Yuan et al, 2000).

Of the three single nucleotide substitution loci (G to A at -995; C to T at -759 and G to C at 697) and one dinucleotide repeat locus at -1,027, haplotype '9' ((Z+2; -995G; -759C and -697G) was associated with obesity and haplotype 2 (Z-6; -995G; -759C and -697C) was more common in non-obese subjects. Haplotype 3 (Z-6; -995A; -759T and -697C) was associated with 'leanness' and the absence of diabetes. In view of this finding, it was hypothesised by the researchers that the C to T -759 promoter polymorphism might be relevant to the potential side-effect of weight gain for patients on anti-psychotics.

A subsequent study therefore investigated whether weight gain in first episode psychotic patients is associated with this polymorphism (Reynolds et al, 2002). As predicted, significantly less weight gain was recorded in patients with the -759T variant allele than in those without the allele. It would therefore seem that this polymorphism has more evidence for its association with anti-psychotic induced weight gain than the cys23ser polymorphism investigated in our study on possible functional differences between the two alleles (Chapter Five).

A further polymorphism relating to the 5-HT_{2C} gene has been identified in the 3'-untranslated region of the 5-HT_{2C} gene (2831T>G) which is approximately 100 kb from

the Cys23Ser variant site (Song et al, 1999). As we did not, in our 5-HT_{2C} cys23ser function study, show any significant differences on m-CPP induced measures, or correlation of either the cysteine or serine substituted receptors with SSRI response in Chapter 4, it is hoped that the newly reported polymorphism might relate not only to weight gain but also to the clinical response of depressed patients to SSRIs.

However, as 5-HT_{2C} receptor heterogeneity derives from a minimum of three possible sources, including allelic variation (as discussed above), two further lines of investigation are also now underway, namely RNA alternative splicing and RNA editing and these have been investigated in some initial studies. Messenger RNA editing of the 5-HT_{2C} receptor was previously noted to occur, with the conversion of one to four adenosines to inosines in rat studies, leading to a change in up to three out of the five codons in the second intra-cellular loop.

It has now been established that the same process occurs in humans, where an editing site exists in the middle codon, resulting in 6 additional isoforms. Whereas the receptor binding affinity was conserved in the rat studies, in human isoforms binding affinity is reduced, with full agonists being the most affected and antagonists showing no effect (Fitzgerald et al, 1999).

In studies involving psychiatric patients, one study has shown reduced RNA editing in the frontal cortex of the brains of schizophrenic patients in relation to control healthy subjects as well as variations in the expression of isoforms (Sodhi et al, 2001), while in another investigation no changes were seen in either schizophrenic patients or those with

major depression (DSM-4), in comparison with healthy controls (Niswender et al, 2001). However, in the latter study, individuals from both patient groups who had committed suicide exhibited a significant increase in editing at one specific site (The 'A' site).

Although the positive correlation of treatment response in schizophrenic patients to clozapine and possession of the 5-HT_{2C}ser substitution was a significant aspect of the background to the m-CPP study in the 5-HT_{2C} variant groups, there is now evidence that the efficacy of clozapine may relate to factors other than serotonin receptor subtypes. Recent findings have again implicated dopamine receptors specifically in models of response to clozapine, rather than models invoking ratios of different neurotransmitters which are blocked by clozapine. One current line of investigation relates to the speed at which clozapine detaches from the dopamine receptor, in the so-called 'fast off' model (Seeman et al, 1999), where atypical anti-psychotic drugs are seen to dissociate more rapidly than typical anti-psychotics from D₂ receptors.

While there are new directions referred to above in the attempt to unravel the relevance of 5-HT_{2C} blockade to the treatment of schizophrenia, it is less clear how the receptor relates to the efficacy of anti-depressants. In Chapter 3 of this thesis evidence was presented for a reduced response of the receptors to m-CPP after a three week treatment period on paroxetine. While this could be explained as the attempt by the neurotransmitter system to maintain homeostasis, it may be linked to the onset of anti-depressant efficacy, some one to two weeks after treatment is commenced. Although work on the 5-HT_{1A} auto-receptor also showed a gradual desensitisation following SSRI

treatment (Sargent et al, 1997), subsequent studies aiming to induce an earlier reduction in function pharmacologically by the use of 5-HT_{1A} antagonists, as investigated pharmacologically, (Blier and Bergeron, 1998), have not been consistently successful clinically. The delayed onset of effect with anti-depressants therefore continues to be investigated further.

Although the role of 5-HT_{2C} receptor modulation in antidepressant efficacy continues to be unclear, it should be noted that a number of antidepressant drugs, notably mirtazapine have potent antagonist properties at 5-HT_{2C} receptors. Animal studies suggest that this property might be associated with increased dopamine release in mesocortical regions. It is also possible, however, that differing effects of antidepressants on 5-HT_{2C} receptors may contribute more to side effect profile (for example – sleep disturbance and aspects of sexual dysfunction) than therapeutic efficacy. Clinical antidepressant trials with selective 5-HT_{2C} receptor agonists and antagonists might enable this question to be resolved.

In Chapter 4 of this thesis, no correlation was found between SSRI response and either the serotonin transporter promoter polymorphism or the 5-HT_{2C} cys23ser polymorphism. Although a number of studies have considered the cys23ser polymorphism in anti-psychotic response in schizophrenia, no previous studies have reported on the polymorphism in relation to response to anti-depressants. It is therefore not clear whether our finding is a valid negative or a chance finding until replication studies have been conducted. We were also unable to replicate the previously reported excess of 's' serotonin transporter promoter alleles in patients with major depression (DSM-IV), in comparison with samples from individuals who do not suffer from major depression.

The clomipramine finding is of significant relevance to the whole area of possible genetic contributions to anti-depressant response and could be usefully advanced with a larger sample and the link of weight gain and therapeutic response could also be extended with anti-depressants such as mirtazepine which has a 5-HT_{2C} antagonist effect.

7.2 FUTURE RESEARCH

As a different 5-HT_{2C} polymorphism to the one studied in this thesis has now been more closely associated with anti-psychotic induced weight gain it will be important to re-analyse the findings in Chapter Five according to the sample split dictated by possession or absence of the -759T variant, when the remaining samples can be genotyped. A positive link with m-CPP - induced hypophagia would lend added weight to the above explanatory framework and suggest that the -759T variant is protective against induced weight gain. A finding such as this would have both clinical and research implications as genotyping prior to treatment would enable clinicians to avoid placing genetically vulnerable patients, at risk of Type 2 Diabetes Mellitus or raised cholesterol and triglycerides, on antipsychotic drugs such as olanzapine. In view of gender differences seen in other studies using m-CPP, it would be important to repeat the tests of 5-HT_{2C} functionality in males. This is also important in view of the X-linkage of the cys23ser gene. Furthermore, as the cys/ser and ser/ser groups were pooled in the initial analysis according to a serine dominant model, it would be important to investigate serine homozygote women as a separate group. Although the analysis was carried out

considering this group alone, there were too few individuals represented in the cell for reliable statistics to be derived.

In view of the above mentioned information on schizophrenia and increasing evidence on the need for anti-depressant medication to be taken by many patients on a long term basis, further clarification of the determinants of response remains an important aim in psychopharmacological research.

APPENDICES

APPENDIX 1: BECK DEPRESSION INVENTORY (Beck et al., 1961)

On this questionnaire are groups of statements. Please read each group of statements carefully, then pick out the one which best describes the way you have been feeling in *the past week including today*. Circle the number beside the statement you have picked. If several statements apply equally well, circle each one. Be sure to read all the statements in each group before making your choice.

- | | | |
|---|---|---|
| 1 | 0 | I do not feel sad |
| | 1 | I feel sad |
| | 2 | I am sad all the time and cannot snap out of it |
| | 3 | I am so sad or unhappy that I can't stand it |

- | | | |
|---|---|---|
| 2 | 0 | I am not particularly discouraged about the future |
| | 1 | I feel discouraged about the future |
| | 2 | I feel I have nothing to look forward to |
| | 3 | I feel that the future is hopeless and that things cannot improve |

- | | | |
|---|---|------------------------------|
| 3 | 0 | I do not feel like a failure |
|---|---|------------------------------|

- 1 I feel I have failed more than the average person
- 2 As I look back on my life, all I can see is a lot of failures
- 3 I feel that I am a complete failure as a person

- 4 0 I get as much satisfaction out of things as I used to
- 1 I don't enjoy things the way I used to
- 2 I don't get real satisfaction out of anything any more
- 3 I am dissatisfied or bored with everything

- 5 0 I don't feel particularly guilty
- 1 I feel guilty a good part of the time
- 2 I feel quite guilty most of the time
- 3 I feel guilty all of the time

- 6 0 I don't feel I am being punished
- 1 I feel I may be punished
- 2 I expect to be punished
- 3 I feel I am being punished

- 7 0 I don't feel disappointed in myself
- 1 I am disappointed in myself
- 2 I am disgusted in myself
- 3 I hate myself

- 8 0 I don't feel I am worse than anyone else
- 1 I am critical of myself for my weakness and mistakes
- 2 I blame myself all the time for my faults
- 3 I blame myself for everything bad that happens

- 9 0 I don't have thoughts of killing myself

- 1 I have thoughts of killing myself but I would not carry them out
- 2 I would like to kill myself
- 3 I would kill myself if I had the chance

- 10
- 0 I don't cry any more than usual
 - 1 I cry more than I used to
 - 2 I cry all the time now
 - 3 I used to be able to cry but now I can't cry even though I want to

- 11
- 0 I am no more irritated now that I ever am
 - 1 I get annoyed or irritated more easily than I used to
 - 2 I feel irritated all the time now
 - 3 I get irritated now by things that didn't used to irritate me

- 12
- 0 I have not lost interest in other people
 - 1 I am less interested in other people than I used to be
 - 2 I have lost most of my interest in other people
 - 3 I have lost all interest in other people

- 13
- 0 I make decisions as well as I ever could
 - 1 I put off making decisions more than I used to
 - 2 I have greater difficulty in making decisions than before
 - 3 I can't make decisions at all anymore

- 14
- 0 I don't feel I look any worse than I used to
 - 1 I am worried that I am looking old or unattractive
 - 2 I feel there are permanent changes in my appearance that make me look old or unattractive
 - 3 I believe that I look ugly

- 15 0 I can work as well as before
 1 It takes an extra effort to get started at doing something
 2 I have to push myself hard to do anything
 3 I can't do any work at all

- 16 0 I can sleep as well as usual
 1 I don't sleep as well as I used to
 2 I wake up 1-2 hours earlier than usual and find it hard to get back to sleep
 3 I wake up several hours earlier than usual and cannot get back to sleep

- 17 0 I don't get more tired than usual
 1 I get tired more easily than I used to
 2 I get tired from doing almost anything
 3 I am too tired to do anything

- 18 0 My appetite is no worse than usual
 1 My appetite is not as good as it used to be
 2 My appetite is much worse now
 3 I have no appetite at all any more

- 19 0 I haven't lost much weight lately
 1 I have lost more than 5 pounds
 2 I have lost more than 10 pounds
 3 I have lost more than 15 pounds

- 20 0 I am no more worried about my health than usual
 1 I am worried about aches and pains, upset stomach and constipation
 2 I am very worried about physical problems and it is hard to think of much else
 3 I am so worried about my physical problems that I can't think of anything else

- | | | |
|----|---|--|
| 21 | 0 | I have not noticed any recent change in my interest in sex |
| | 1 | I am less interested in sex than I used to be |
| | 2 | I am less interested in sex now |
| | 3 | I have lost interest in sex completely |

APPENDIX 2: HAMILTON DEPRESSION RATING SCALE (Hamilton, 1960)

NAME _____

DATE _____

SCORING GUIDE: 0-4: 0 = ABSENT, 1 = MILD-TRIVIAL; 2,3 = MODERATE

4 = SEVERE/INCAPACITATING

0-2: 0 = ABSENT, 1 = SLIGHT/DOUBTFUL, 2 = CLEARLY PRESENT

1. Depressed mood	1	Indicated on questioning	
	2	Spontaneously reported	
	3	Obvious at interview	
	4	Only feeling reported	
2. Guilt	1	Self reproach	
	2	Ideas/rumination of guilt	
	3	Delusions of guilt	
	4	Persecutory hallucinations	
3. Suicide	1	Feels life not worth living	
	2	Wishes were dead	
	3	Suicidal ideas/gestures	
	4	Attempts at suicide	
4. Initial insomnia more than ½ an hour	1	Occasional	
	2	Persistent	
5. Middle insomnia	1	Restless and disturbed during night	
	2	Waking / getting out of bed	
6. Late insomnia	1	Wakes but returns to sleep/Unable to fall asleep again	
7. Work and interests	1	Fatigue/feelings of	

		incapacity 2 Loss of interest 3 Decrease in time/productivity 4 Stopped working because of illness	
8. Retardation		1 Slight 2 Obvious 3 Interview difficult 4 Complete stupor	
9. Agitation		1 Fidgetiness 2 Playing with hands/hair 3 Moving about/can't sit still 4 Hand wringing / hair pulling	
10. Psychic anxiety		1 Tension, irritability 2 Worrying over minor matters 3 Apprehensive in body language 4 Fears spontaneously reported	
11. Somatic anxiety		1 Mild 2 Moderate 3 Severe 4 Incapacitating	
12. Gastrointestinal symptoms		1 Loss of appetite 2 Marked difficulty in eating	

13. Somatic symptoms	1	Heaviness / aches / fatigue	
	2	Clear cut symptoms	
14. Genital symptoms	1	Mild	
	2	Severe	
15. Hypochondriasis	1	Bodily self absorption	
	2	Preoccupation with health	
	3	Frequent complaints/ requests for help	
	4	Delusions	
16. Loss of weight	1	Probable	
	2	Definite	
17. Insight	1	Partial	
	2	Clear Loss	
	TOTAL SCORE		
18. Diurnal variation	a.m. or p.m.	mild or severe	
19. Depersonalization	Mild		
	moderate		
	severe		
	incapacitating		
20. Paranoid symptoms	Suspicious		
	ideas of reference		
	delusions		
21. Obsessional symptoms	Mild		
	severe		

APPENDIX 3: VISUAL ANALOGUE SCALES

PLEASE EITHER PLACE A MARK ON THE LINE OR
CIRCLE THE NUMBER WHICH BEST INDICIATES HOW
YOU FEEL RIGHT NOW

NAME:

SESSION:

DATE:

TIME:

I feel –

0 10 20 30 40 50 60 70 70 80 90 100

-----|

Not at all happy

Very happy

0 10 20 30 40 50 60 70 70 80 90 100

-----|

Not at all drowsy

Very drowsy

0 10 20 30 40 50 60 70 70 80 90 100

-----|

Not at all anxious

Very anxious

0 10 20 30 40 50 60 70 70 80 90 100

-----|

Not at all light-headed

Very light-headed

0 10 20 30 40 50 60 70 70 80 90 100

-----|

Not at all nauseous

Very nauseous

APPENDIX 4: 5-HT_{2C} POLYMORPHISM m-CPP CHALLENGE STUDY. RECORD OF FOOD PREFERRED.

FOOD PREFERENCE

NAME:

DATE:

Please rate the following foods according to your preferences, using the score below.

1	2	3	4	5	6	7	8	9	10
Dislike			Neutral				Like		
Extremely							Extremely		

Section 1: Bread

_____ White
_____ Soft grain (mighty white)
_____ Wholemeal

Section 2: Sandwich fillings

_____ Tuna
_____ Cheddar Cheese
_____ Chicken (wafer thin American style)
_____ Ham (wafer thin American style)

Section 3: Additional fillings

_____ Cucumber
_____ Mayonnaise

APPENDIX 5: 5-HT_{2C} POLYMORPHISM m-CPP CHALLENGE STUDY. RECORD OF FOOD CONSUMED.

Results Sheet

Name:

Date:

Familiarisation Meal (2nd choice of bread & filling)

Bread:

Filling:

Mayo: Yes/No

Cucumber: Yes/No

Time taken:

Number quarters consumed:

Kcal/Quarter:

Total Kcal consumed:

Test Meal 1 (1st choice of bread & filling)

Date:

Bread:

Filling:

Mayo: Yes/No

Cucumber: Yes/No

Time taken:

Number quarters consumed:

Kcal/Quarter:

Total Kcal consumed:

Test Meal 2 (1st choice of bread & filling)

Date:

Bread:

Filling:

Mayo: Yes/No

Cucumber: Yes/No

Time taken:

Number quarters consumed:

Kcal/Quarter:

Total Kcal consumed:

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