PHARMACOGENETIC ANALYSIS OF SEROTONIN RECEPTORS AND CLINICAL RESPONSE TO CLOZAPINE IN SCHIZOPHRENIA PATIENTS

by

Mario Masellis

A thesis submitted in conformity with the requirements for the degree of Master of Science in the Graduate Department of Pharmacology, University of Toronto

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Pharmacogenetic Analysis of Serotonin Receptors and Clinical Response to Clozapine in Schizophrenia Patients

Master of Science 1997

Mario Masellis

Graduate Department of Pharmacology, University of Toronto

ABSTRACT

Clozapine, the prototype of atypical antipsychotic drugs, is the best means available for the treatment of schizophrenia patients refractory or intolerant to typical antipsychotic therapy. Its pharmacological profile is quite unique as it possesses affinity for receptors from many different neurotransmitter systems. The serotonergic system has been implicated in the mechanism of action of clozapine. In particular, clozapine's affinity at 5-HT2A, 5-HT2C, 5-HT1A, 5-HT6, and 5-HT7 receptors may contribute to its many unique clinical attributes.

Using a pharmacogenetic approach in 185 schizophrenia patients who have been prospectively assessed for clozapine response, we have examined the hypothesis that polymorphisms in the 5-HT2A (HTR2A), and 5-HT2C (HTR2C) genes are involved in its variable response. A -1437 A→G polymorphism in the putative promoter, and a silent T→C 102 substitution in HTR2A were in almost complete linkage disequilibrium, and neither was associated with response (T→C 102 allele: $\chi^2=0.02$, 1 df, $p=0.90$; genotype: $\chi^2=0.02$, 2 df, $p=0.99$). A his452tyr HTR2A polymorphism was found to be significantly associated with clozapine response (his452tyr allele: $\chi^2=6.43$, 1 df, $p=0.01$; genotype: $\chi^2=6.54$, 2 df, $p=0.04$). No HTR2A haplotype was associated with response. Inter-ethnic
differences were observed in the frequencies of the cys23ser HTR2C polymorphism. This polymorphism was not significantly associated with response in either of the ethnic groups (Caucasian and African-American genotype: $\chi^2=3.46$, 2 df, $p=0.18$; $\chi^2=0.31$, 2 df, $p=0.86$, respectively). Although replication is required, the overall results suggest that the his452tyr HTR2A polymorphism may be involved in clozapine response.

Polymorphisms in the 5-HT1A (HTR1A), 5-HT6 (HTR6), and 5-HT7 (HTR7) receptor genes were also examined in this sample. The pro16leu HTR1A polymorphism was not observed in our sample; all individuals genotyped were pro/pro 16 homozygotes. With respect to the pro279leu HTR7 polymorphism, one Caucasian male responder to clozapine was observed to be heterozygous (pro/leu 279 genotype). This individual was clinically similar to the other clozapine responders. No evidence for either an allelic or genotypic association of the T→C 267 HTR6 polymorphism with response to clozapine was found in our sample (allele: $\chi^2=0.06$, 1 df, $p=0.80$; genotype: $\chi^2=0.06$, 1 df, $p=0.80$).

Overall, our results are consistent with the hypothesis that genetic variation in the 5-HT2A receptor is involved in the phenotype of clozapine response. A polymorphism which causes an amino acid substitution in the 5-HT2A receptor was found to be significantly associated with response to this atypical antipsychotic drug. Further functional investigation of this polymorphism is warranted as there is preliminary evidence suggesting differences between the variants in their sensitivity to serotonin.
ACKNOWLEDGEMENTS

I would like to thank first and foremost my supervisors, Dr. James L. Kennedy and Dr. Werner Kalow. Dr. Kalow first stimulated my interest in and provided me with my first exposure to research, and his guidance, suggestions, and criticisms over the years have been exceptional. These have helped me to critically analyze and think about the scientific problems which I have encountered, and have significantly helped to develop and shape my scientific abilities.

Almost three years ago, I had just finished my undergraduate degree and was looking for a M.Sc. supervisor, particularly in the field of neuropsychopharmacology. After talking to several people working in this field without success, I was referred to the Neurogenetics section at the Clarke Institute of Psychiatry headed by Dr. Jim Kennedy. Coming from purely a pharmacological background, the thought of learning a new field, especially genetics, caused a great deal of anxiety within me. Despite this, I met with Dr. Kennedy for about an hour, and discussed with him a potential project employing molecular genetic techniques to examine psychopharmacological drug response.

My experiences in Dr. Kennedy’s lab have been, to say the least, outstanding. The project that I have been working on has taught me a great deal in the fields of psychiatry, and genetics and these have been successfully integrated with my pharmacological background. I would like to thank Dr. Kennedy for his exceptional guidance, impeccable patience, excellent suggestions and criticisms, and thought provoking discussions over the past few years. These have all helped to contribute to and develop my scientific abilities. I would especially like to thank Dr. Kennedy for providing me with many opportunities in
research, of which there are too many to describe. Thank you for trusting and having faith in my abilities; sometimes I was even doubtful of these myself.

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Last but not least, I would like to thank my parents. I greatly appreciate their continued support and inspiration and I am indebted to their patience over the past few years, especially during the preparation of this thesis.
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ABBREVIATIONS

δ  disequilibrium coefficient
φ  phi coefficient
α  probability associated with making a type I error
β  probability associated with making a type II error
χ²  chi-square statistic
γ¹²P  γ³²P phosphate
°C  degrees Celsius
µL  microlitre
µM  micromolar
1-β  power
5-HIAA  5-hydroxyindoleacetic acid
5-HT  5-hydroxytryptamine or serotonin
5-HT1A  serotonin 1A receptor
5-HT2A  serotonin 2A receptor
5-HT2C  serotonin 2C receptor
5-HT6  serotonin 6 receptor
5-HT7  serotonin 7 receptor
A  adenosine
A.P.A.  American Psychiatric Association
ACh  acetylcholine
Ah  aromatic hydrocarbon receptor
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<tr>
<td>ASO</td>
<td>allele-specific oligonucleotide</td>
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<td>bp</td>
<td>base pair</td>
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<td>BPRS</td>
<td>Brief Psychiatric Rating Scale</td>
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<td>C</td>
<td>cytosine</td>
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<td>Ca²⁻</td>
<td>calcium</td>
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<td>CGI</td>
<td>Clinical Global Impressions Scale</td>
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<td>CI</td>
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<td>df</td>
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<td>dGTP</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DRD4</td>
<td>dopamine D4 receptor gene</td>
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<td>dTTP</td>
<td>2'-deoxythymidine-5'-triphosphate</td>
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<td>EH</td>
<td>equilibrium haplotype</td>
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<td>EPS</td>
<td>extrapyramidal symptoms</td>
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<td>F</td>
<td>F-statistic</td>
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<td>G</td>
<td>guanosine</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<td>GAS</td>
<td>Global Assessment Scale</td>
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<td>his</td>
<td>histidine</td>
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<td>HRR</td>
<td>haplotype relative risk</td>
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<td>HSD</td>
<td>honestly significant difference</td>
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<td>human serotonin 2C receptor gene</td>
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<td>human serotonin 6 receptor gene</td>
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<td>HTR7</td>
<td>human serotonin 7 receptor gene</td>
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<td>HVA</td>
<td>homovanillic acid</td>
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<td>leu</td>
<td>leucine</td>
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<td>LSD</td>
<td>lysergic acid diethylamide</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>mCPP</td>
<td>meta-chlorophenylpiperazine</td>
</tr>
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<td>MgCl₂</td>
<td>magnesium chloride</td>
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<td>mL</td>
<td>millilitre</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>n</td>
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<td>ng</td>
<td>nanograms</td>
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<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>p</td>
<td>probability value</td>
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<td>P.E.T.</td>
<td>positron emission tomography</td>
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<td>P₀</td>
<td>proportion of control individuals with risk allele</td>
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<td>restriction fragment length polymorphism</td>
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<td>sodium dodecyl sulfate</td>
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<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
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<td>SSCP</td>
<td>single-stranded conformational polymorphism</td>
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<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
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<td>thymidine</td>
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<td>tardive dyskinesia</td>
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tyr tyrosine
UV ultraviolet light
VTA ventral tegmental area
LIST OF THESIS PUBLICATIONS

ARTICLES:

a) Published:


b) In press:


c) Submitted:


BOOK CHAPTERS:


INVITED LECTURES:


ORAL PRESENTATIONS:

ABSTRACTS:


**GRANTS (funded):**

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*Primary Investigator:* Levitan RD

*Co-Investigators:* Masellis M, Kennedy SH, Kaplan AS, Vaccarino F, Woodside B

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*Amount:* $59,838 per year for 2 years
1.0 General Introduction
1.0 General Introduction

1.1 Schizophrenia

Schizophrenia is perhaps the most devastating of the psychiatric disorders affecting approximately 1% of the population world-wide. It is a chronic disease beginning in young adulthood with onset typically occurring in the late teens to mid-thirties. Individuals suffering from schizophrenia experience problems in one or more major areas of functioning such as work or education, interpersonal relations, and/or self-care. Approximately 10% of schizophrenics commit suicide.

The characteristic symptoms of schizophrenia can be broken down into two broad clusters, the positive and negative symptoms. The positive symptoms involve an excess or distortion of certain normal functions while the negative symptoms are conversely distinguished by a diminution or loss of other normal functions.

The positive symptom cluster can be further subdivided into two dimensions, "psychotic" and "disorganized". The "psychotic dimension" encompasses both distortions or exaggerations of inferential thinking (delusions), and perception (hallucinations). The "disorganized dimension" includes distortions in language and communication (disorganized speech), and behavioural monitoring (grossly disorganized or catatonic behaviour). The negative symptom cluster is characterized primarily by problems in the reduction of range and intensity of emotional expression (affective flattening), fluency and productivity of thought and speech (alalia), and initiation of goal-directed behaviour (avolition). It is thought that there are distinct neural mechanisms underlying each of these symptom clusters (adopted from A.P.A., 1994).
1.2 The dopamine hypothesis of schizophrenia

The dopamine hypothesis of schizophrenia postulates that overactivity at dopaminergic synapses in the central nervous system (CNS), particularly the mesolimbic system, causes the psychotic symptoms of schizophrenia (Carlsson, 1988; Meltzer and Stahl, 1976; Snyder, 1973; Snyder, 1976). This hypothesis was initially based on the following observations: antipsychotic drugs caused motor side effects (extrapyramidal symptoms - EPS), such as tremor and rigidity, that were clinically similar to the hypodopaminergic symptoms of Parkinson's disease (reviewed by Seeman, 1995); and psychostimulant drugs (dopamine mimetics) can elicit psychotic symptoms and these could be attenuated by antipsychotic agents (Lieberman et al., 1987). Perhaps the most compelling evidence in support of the dopamine hypothesis of schizophrenia stems from the fact that the in vitro affinities of antipsychotic agents for dopamine D2 receptors correlated well with their clinical potencies (Creese et al., 1976; Seeman et al., 1975; 1976; 1992). In summary, the ability of antipsychotic drugs to block dopamine D2 receptor sites is thought to reduce the dopaminergic overactivity in the mesolimbic system and thus alleviate the psychotic symptoms of schizophrenia. The term typical antipsychotic will now be used to refer to antipsychotics that possess predominantly D2 receptor blocking properties and cause catalepsy in rodents and EPS in humans.

1.3 The serotonin hypothesis of schizophrenia

The serotonin hypothesis of schizophrenia was initially provided by Wooley and Shaw (1954), and Gaddum and Hameed (1954) and was based on the observation that lysergic acid diethylamide (LSD), initially thought to be a serotonin (5-HT) antagonist,
possessed potent hallucinogenic properties in humans. More recent studies confirmed that LSD was actually acting as a partial agonist at 5-HT2A/2C receptors (Glennon, 1990). One of the major problems with this hypothesis was that LSD caused primarily visual hallucinations which are quite rare in schizophrenia; auditory hallucinations are by far the most common. This, in addition to the strength of the dopamine hypothesis of schizophrenia, caused a shift away from the role of serotonin in schizophrenia.

It was not until recent years, with the re-introduction of the atypical antipsychotic, clozapine (discussed below), that there was a resurgence of the serotonin hypothesis of schizophrenia. Although it is beyond the scope of this thesis to go into the details that support this hypothesis, a brief overview will be provided. Bleich et al. (1988), Roth and Meltzer (1995), and Breier (1995) have provided extensive reviews of the literature and have concluded that there is a large amount of information implicating a role for serotonin in the pathophysiology and treatment of schizophrenia. The evidence is as follows: 1.) alterations in levels of 5-HT receptors have been found in schizophrenia; 2.) neuroendocrine challenge studies suggest a subsensitivity of some types of serotonin receptors; 3.) many typical and atypical antipsychotic agents bind with high affinity to 5-HT receptors; 4.) changes in central serotonergic systems have been correlated with deficit symptoms of schizophrenia; 5.) pharmacological studies in animals are consistent with the notion that the 5-HT system may serve as one of the regulators of dopaminergic tone in vivo (reviewed by Roth and Meltzer, 1995).
1.4 The prototype of atypical antipsychotics: Clozapine

Clozapine, the prototype of atypical antipsychotic drugs, has been reported to have a variety of clinical advantages for the treatment of schizophrenia compared to typical or traditional antipsychotic drugs. These include: 1) efficacy in treatment-refractory or intolerant schizophrenia; 2) improvement in negative symptoms; 3) improvement in some types of cognitive function; 4) almost complete absence of extrapyramidal side effects (EPS); 5) virtually no ability to cause tardive dyskinesia (TD) after chronic use; and 6) no increase in serum prolactin levels (reviewed by Meltzer, 1995a).

Clozapine has a very unique pharmacological profile. It possesses affinity for a variety of receptors from several different neurotransmitter systems. These include the dopamine D1-D4, 5-HT1A, 5-HT2A, 5-HT2C, 5-HT3, 5-HT6, 5-HT7, α1-adrenergic, muscarinic M1-M5, and histaminergic H1 and H3 receptors. Please refer to Ashby and Wang (1996) for a summary of clozapine’s Ki values at the aforementioned receptor sites.

Based on clozapine’s broad pharmacological profile, many hypotheses regarding its mechanism of action have been generated. Clozapine has low affinity for dopamine D2 receptors and this may be involved in its decreased ability to induce EPS (Farde et al., 1992; Nordström et al., 1995). It also has a relatively high affinity for dopamine D1 receptors in comparison to typical antipsychotic drugs and this has been hypothesized to be involved in clozapine’s efficacy against negative symptoms as well as its ability to prevent TD (reviewed by Kinon and Lieberman, 1996). The D4 hypothesis is based on the fact that clozapine has a 10-fold higher affinity for the dopamine D4 receptor than for the D2 receptor (Van Tol et al., 1991; Seeman, 1992). Clozapine’s high affinity for
muscarinic cholinergic receptors has been implicated in its mechanism of action regarding decreased EPS (Miller and Hiley, 1974; Snyder et al., 1974).

These are just a few of the hypotheses that have been proposed to explain clozapine's 'atypical' clinical effects. It is likely that the mechanism of action of clozapine is not limited to one specific pharmacological interaction. Rather its affinity at multiple receptor sites is responsible for its unique clinical properties. This idea is feasible considering the intricate interactions that take place between the different neurotransmitter pathways in the central nervous system and the role that they play in determining complex human behaviour. It is beyond the scope of this thesis to provide a comprehensive review of the hypothesized mechanisms of action of clozapine; these have been discussed in detail elsewhere (Meltzer, 1994; Kinon and Lieberman, 1996; Ashby and Wang, 1996). The serotonin-dopamine interaction hypothesis of clozapine's mechanism of action will now be thoroughly discussed as this will provide the basis for the central hypothesis of the thesis.

1.5 Interaction between the dopaminergic and serotonergic systems

It is known that the dopaminergic and serotonergic systems synapse extensively with each other and that alterations in one system can influence the function of the other system. This section and the next will review the dopaminergic and serotonergic pathways in the brain and the nature of the interaction between them, and explain the putative relevance of this serotonin-dopamine interaction in the mechanism of action of clozapine and atypical antipsychotic drugs.
1.5.A Dopaminergic pathways and their function

There are three dopaminergic pathways that have been implicated in the pathophysiology of schizophrenia: 1.) the nigrostriatal tract, 2.) the mesolimbic tract, and 3.) the mesocortical tract. Neurons originating from the substantia nigra of the midbrain send projections to the striatum to form the nigrostriatal tract. This tract plays a major role in the modulation of motor behaviour. Neurons originating from the ventral tegmental area (VTA) of the midbrain project to limbic and cortical regions forming the mesolimbic and mesocortical tracts, respectively. These tracts are believed to play an important role in cognitive and emotional processes, and the modulation of motivation and reward (reviewed by Le Moal, 1995; Joyce and Meador-Woodruff, 1997).

1.5.B Serotonergic pathways and their function

The serotonergic system of the brain is believed to play a role in a wide range of functions including neuronal development, sleep, motor control, sexual activity, feeding behaviour, mood, aggression, neuroendocrine function, and cognitive function (reviewed by Siever et al., 1991). However, it does not appear to be essential for any of them (Jacobs and Azmitia, 1992). The reason for this can be explained by the concept that the serotonin system "exerts a tonic modulatory influence on its widespread targets" (Jacobs and Azmitia, 1992).

The serotonergic system is probably the most widely distributed of the neurotransmitter systems studied. Although the serotonergic cell bodies are restricted to discrete nuclei in the midbrain, their fibers innervate almost every area of the brain. The major serotonergic tracts arise predominantly from two discrete midbrain nuclei: the
dorsal raphe and the median raphe. The median raphe nucleus projects to the limbic regions, while the dorsal raphe nucleus sends projections via the median forebrain bundle to the cortical and striatal regions (reviewed by Jacobs and Azmitia, 1992).

1.5.C Functions of the serotonin-dopamine interaction

Serotonergic projections to the dopamine cell bodies of the substantia nigra, which originate as collaterals of the dorsal raphe-striatal neurons, inhibit the firing of the nigral dopamine neurons. The inhibitory action of the raphe-nigral serotonin neurons appears to be mediated by 5-HT2 receptor sites located on the somatodendritic surface of the nigral dopamine neurons. Anatomical or chemical lesions disrupting raphe-nigral projections, 5-HT1A agonists, through their action as somatodendritic autoreceptors, that decrease the firing of raphe-nigral neurons, or 5-HT2 antagonists that block the effect of the raphe-nigral serotonin neurons all produce a functional disinhibition of the nigrostriatal dopaminergic pathway (reviewed by Kapur and Remington, 1996). See Figure 1 for a schematic representation of this serotonin-dopamine interaction.

The dopaminergic projections from the substantia nigra to the striatum are also inhibited by the serotonergic raphe-striatal projections (reviewed by Kapur and Remington, 1996) (see Figure 1). Once again, the effect seems to be mediated by 5-HT2 receptors. These are located on the terminals of the nigrostriatal projections. It is thought that stimulation of these 5-HT2 receptors may decrease release of dopamine or decrease its synthesis in the nigrostriatal terminals. Agents that increase serotonin release from these raphe-striatal projections or serotonin agonists applied directly to the striatal region decrease the release of endogenous dopamine. Lesions of this raphe-striatal tract or
blockade of 5-HT2 receptors blocks this inhibitory action of serotonin on the striatal dopamine system causing increases in levels of striatal dopamine, i.e. disinhibition.

Similar interactions between the dopamine and serotonin systems are believed to modulate limbic and cortical dopaminergic pathways as well. It has been observed that clozapine induces an increased turnover of dopamine in the prefrontal cortex of rodents which is thought to occur via blockade of 5-HT2 receptors; this effect is not observed with typical antipsychotics (Moghaddam and Bunney, 1990; Moghaddam, 1994). It should be pointed out that the serotonin system interacts with the GABA and cholinergic systems as well, and thus the ability of serotonin to modulate dopamine may be mediated, indirectly, through its action on these systems.
1.5.D The serotonin-dopamine interaction: Relevance to EPS and negative symptoms

Typical antipsychotic drugs are thought to cause extrapyramidal side effects by blocking dopamine D2 receptors in the striatum and thus reducing dopaminergic function (Farde et al., 1992). Catalepsy induced by traditional antipsychotic drugs is thought to occur via a similar mechanism in animals and thus has been proposed as a model of EPS (Sanberg, 1980). Based on the previous section, inhibition of the serotonergic raphe-nigral and -striatal pathways should enhance the function of the nigrostriatal projections.
and increase dopamine levels at the striatum. This hypothetically should alleviate catalepsy in animals and EPS in humans.

It has been demonstrated in numerous studies (reviewed by Kapur and Remington, 1996) that catalepsy is reduced by manipulations that inhibit the raphe-nigral and -striatal serotonergic pathways. Thus lesions of the raphe prevent and ameliorate catalepsy in rodents. 5-HT1A agonists, through their actions as somatodendritic autoreceptors, can reverse and prevent catalepsy in rodents, and EPS in primate models. Also, 5-HT2 antagonists have been reported to prevent and ameliorate catalepsy in animal models.

Kapur and Remington (1996) have also reviewed human studies of EPS and modulation by serotonergic drugs. Several studies have demonstrated that 5-HT2 antagonists have a beneficial effect on extrapyramidal symptoms while selective serotonin reuptake inhibitors (SSRIs) have been shown to induce some forms of EPS.

Another problem with typical antipsychotics is their lack of efficacy in treating the negative symptoms of schizophrenia. Conversely, clozapine and other atypical antipsychotic drugs have been shown to be effective in alleviating negative symptomatology (Breier et al., 1994; Carpenter, 1995a; Kane et al., 1988; Pickar et al., 1992; Rao and Moller, 1994; Tollefson et al., 1997; Tollefson and Sanger, 1997). It has been hypothesized that negative symptoms are the result of hypodopaminergic function in the prefrontal cortex (Carlsson and Carlsson, 1990; Davis et al., 1991; Deutch, 1992; Weinberger et al., 1986; Weinberger and Berman, 1988; Weinberger and Lipska, 1995). It is plausible that drugs which decrease serotonergic function of the dorsal raphe-cortical projections can lead to a disinhibition of the mesocortical dopaminergic projections, and presumably ameliorate the negative symptoms as a result of increased dopamine levels in
the prefrontal cortex. Several studies have demonstrated that 5-HT2 antagonists are beneficial in the treatment of negative symptoms (reviewed by Kapur and Remington, 1996). Thus, clozapine's mechanism of action with respect to negative symptoms may be, at least in part, mediated by its antagonistic action on 5-HT2 receptors.

1.6 The serotonin-dopamine hypothesis: A putative role in the mechanism of action of clozapine and atypical antipsychotics

Meltzer and colleagues have hypothesized that the clinical advantages of clozapine and other atypical antipsychotics, especially those related to EPS and negative symptoms, may be due to their significantly higher affinity for 5-HT2A receptors relative to their affinity at D2 receptors (Meltzer et al., 1989a; Meltzer and Nash, 1991; Meltzer, 1994; 1995a). This was based on the extensive body of evidence of a serotonin-dopamine interaction, as discussed in the previous section, and on the fact that the one common feature that all atypical antipsychotics shared was a higher relative affinity for 5-HT2A receptors than for D2 receptors (Meltzer et al., 1989a; Meltzer and Nash, 1991). This same feature was not observed for typical antipsychotic drugs as they had either similar affinities at each receptor or a much higher affinity for D2 receptors. 5-HT2C receptors have also been implicated (Canton et al., 1990; Kahn et al., 1993). Further support of this hypothesis comes from in vivo studies using positron emission tomography (PET). Studies have shown that clozapine, risperidone, and olanzapine, all atypical antipsychotics, have a much higher affinity for 5-HT2A receptors than for D2 receptors in schizophrenic patients (Farde et al., 1994; Farde et al., 1995; Goyer et al., 1996; Nordstrom et al., 1995; Nyberg et al., 1993; Nyberg et al., 1997).
Meltzer and colleagues’ hypothesis has been recently re-evaluated by Kapur and Remington (1996). The authors were in accordance with the likely importance of the serotonin-dopamine interaction in the mechanism of atypical antipsychotic drug action. They also supported the concept of a higher relative affinity of 5-HT2A to D2 receptors as being important in this mechanism. However, they suggested that the benefits of atypical antipsychotics, especially those pertaining to reduced EPS, may be lost as the occupancy of D2 receptors becomes too high. It is important to note here that the ratio of 5-HT2A to D2 affinity is fixed, while the relative level of 5-HT2A to D2 antagonism produced by a given drug in vivo is a function of the dose (Kapur and Remington, 1996). In other words, as the dose of a particular drug with a particular ratio of 5-HT2A:D2 antagonism increases, the level of blockade at each receptor site increases proportionately.

EPS has been found to occur with typical antipsychotics when D2 receptor occupancy exceeds a particular threshold, thought to be in the range of 75-80% (Farde et al., 1992). Kapur and Remington (1996) have suggested that the higher relative affinity of 5-HT2A to D2 receptors seen with atypical antipsychotics may provide a protection against EPS when D2 affinity is slightly above the EPS threshold. In this way, a greater antipsychotic activity may be achieved without the problem of EPS. However, as the dose of the atypical antipsychotic is increased to a point where D2 occupancy greatly exceeds the EPS threshold, the protective effect of 5-HT2A blockade may be lost (Kapur and Remington, 1996). A similar mechanism may be working with respect to negative symptomatology although more work is required to elucidate the precise pathways involved.
In summary, Kapur and Remington (1996) have proposed two hypotheses of how clozapine and atypical antipsychotics with a high ratio of 5-HT2A:D2 blockade may work in reducing EPS (refer to Figure 2). The first suggests that 5-HT2A antagonism could increase the release of dopamine from the nigrostriatal tract leading to an increased displacement of antipsychotic from D2 receptors in the striatum. This would shift the D2 receptor occupancy curve to the right, meaning that a higher dose of antipsychotic will be required to cross the EPS threshold (Hypothesis I in Figure 2). Alternatively, 5-HT2A antagonism may increase the EPS threshold by means of an indirect effect on the dopamine system via its interactions with the GABA and cholinergic systems (Hypothesis II in Figure 2). With respect to a hypothesis for negative symptom amelioration by atypicals, 5-HT2A antagonism should increase dopamine release in a hypofunctioning mesocortical system, thus improving negative symptoms. However, it should be cautioned that these hypotheses make many assumptions and for a detailed description of them, please refer to Kapur and Remington (1996). In addition, the serotonin-dopamine interaction can also be modulated by serotonin system proteins other than 5-HT2A receptors. These may include 5-HT1A, 5-HT2C, 5-HT6, and 5-HT7 receptors for which clozapine and other atypical antipsychotics possess affinity.
**Hypothesis I**

5-HT₂ blockade shifts D₂ occupancy curve to the right

**Hypothesis II**

5-HT₂ blockade raises the EPS threshold

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**Figure 2.** Two hypotheses regarding the mechanisms by which 5-HT2 antagonists reduce extrapyramidal symptoms (EPS) (diagram taken from Kapur and Remington, 1996).

Hypothesis I demonstrates how the addition of 5-HT2 antagonism releases endogenous dopamine and shifts the D2 occupancy curve to the right. The solid curve depicts the D2 occupancy curve without 5-HT2 blockade while the dashed curve depicts the D2 occupancy curve under the influence of 5-HT2 antagonism. The EPS threshold remains constant while the dose at which EPS is observed increases because of a rightward shift in the D2 curve. Hypothesis II suggests that the EPS threshold is increased with the addition of a 5-HT2 antagonist without having a direct effect on D2 occupancy. The dose at which the EPS manifests itself is increased, but the D2 occupancy curve remains the same. Both mechanisms can explain how the addition of 5-HT2 antagonism reduces EPS.

### 1.7 Variability in response to clozapine: Polymorphisms in receptor genes

Despite all of clozapine’s unique and advantageous clinical properties, the therapeutic response to this atypical antipsychotic is highly variable among patients. Between 30 to 60% of patients refractory or intolerant to typical antipsychotic therapy respond beneficially to clozapine (reviewed by Bleehen, 1993).

Central pharmacodynamic factors are likely determinants of inter-individual variation in response to clozapine. Several groups have suggested that genetic factors may contribute to differences in pharmacodynamic response to clozapine (Kennedy, 1994; Kerwin et al., 1994; Propping and Nöthen, 1995), and this is the basis for our central hypothesis.
Central Hypothesis: Genetic variation or polymorphism in genes that encode serotonin receptor proteins (5-HT2A, 5-HT2C, 5-HT1A, 5-HT6, and 5-HT7) for which clozapine has affinity could account, at least in part, for a significant amount of the variance observed in its response.

See Chapter 3 for a more detailed discussion of the topic of variability in response to clozapine.

1.8 Research Objective

The overall objective of this thesis is to identify pharmacodynamic genetic predictors of response to clozapine from the serotonin system. This genetic information may aid clinicians in deciding who should receive a particular drug, and it may also help in elucidating the mechanism of action of atypical antipsychotic agents. Ultimately, the genetic information may lead to the design of new, more specific therapeutic agents, and may help to illuminate our understanding of the pathophysiology of schizophrenia.

A genetic association strategy was employed to assess polymorphisms in candidate genes from the serotonin system in a large, well-characterized sample of clozapine-treated schizophrenia patients who were prospectively assessed for response to this atypical antipsychotic.

The polymorphisms examined in this thesis may be one of three types: 1.) they may occur within the coding regions of the genes and might therefore encode receptor proteins which are structurally and potentially functionally different from each other; thus they may be directly associated with the occurrence of the phenotype of response to clozapine; 2.) if
the polymorphisms do not lead to structurally different proteins, then they can still be used as genetic markers which may occur nearby on the same strand of DNA, and thus identify other susceptibility sites (sites which may be directly associated with the occurrence of response); 3) polymorphisms may also occur within the regulatory regions of a gene and may therefore lead to differences in the expression of their gene products. These expression differences may be associated with the occurrence of the response phenotype as well.

In terms of population genetics, association studies, similar to the ones presented in this thesis, compare affected and unaffected groups of unrelated individuals to identify a marker that is in linkage disequilibrium (i.e. markers at trait loci that are not yet at equilibrium in the population and so do not vary randomly) with the susceptibility site for the trait in question, i.e. response. Each of the chapters will now be outlined and briefly discussed with respect to their overall contribution to the central hypothesis of this thesis.

1.8.A Description of Chapters 2 and 3

In review of sections 1.5-1.6, there is a great deal of evidence suggesting that 5-HT2 receptor antagonism plays a role in increasing dopamine levels in the striatum and prefrontal cortex and this, in turn, is thought to reduce EPS and negative symptomatology in schizophrenia, respectively. Clozapine is an antagonist at and possesses a high affinity for 5-HT2A/2C receptors and this may contribute to some of clozapine’s unique clinical effects.

Chapter 2 entitled ‘Genetic variation of 5-HT2A receptor and response to clozapine’ has examined a silent thymidine to cytosine (T→C) polymorphism at position
102 in the first exon of the 5-HT2A receptor gene (HTR2A) in our initial sample of clozapine-treated patients. In this sample, we found no evidence of a genetic association of the variant with response, however, the sample size was relatively small (n=126) and thus we were limited with respect to our power to detect a significant difference. As a result, we combined our sample with that of Arranz et al. (1995a) and overall found evidence for an association of the T→C 102 HTR2A variant with clozapine response. This preliminary finding stimulated us to examine other polymorphisms at the HTR2A locus in a larger clozapine response sample.

Chapter 3 entitled ‘Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients’ provides a comprehensive assessment of the role of polymorphic variations in both the 5-HT2A and 5-HT2C receptor genes and prediction of response to clozapine. Three polymorphisms in HTR2A were examined, as well as one in the serotonin 2C receptor gene (HTR2C). This chapter significantly extends the work of the preceding chapter in a larger sample of patients all of whom have been prospectively characterized for response to clozapine. An extensive review of the literature and a critical discussion of the current methods being employed for pharmacogenetic analysis of clozapine response are provided. Several suggestions are made for future work being conducted in this field.

1.8.B Description of Chapter 4

All of the genetic work completed to date regarding the role of serotonin receptors in the mechanism of action of clozapine has focused on 5-HT2A/2C receptors. However, clozapine possesses affinity for other serotonin receptors and these may be involved in its
mechanism of action. In this chapter, polymorphisms in other serotonergic candidate genes are analyzed with respect to their putative role in clozapine response. These include the 5-HT1A, and the recently cloned 5-HT6 and 5-HT7 receptor genes. A rationale for their role in the mechanism of action of clozapine is provided, and their putative roles are assessed using a genetic association strategy.
2.0 Genetic variation of 5-HT2A receptor and response to clozapine


As published in: Lancet (1995); 346: 1108 [letter]

Mario Masellis performed the genotyping of the patient sample, the genetic data analysis, and the writing of the letter. All clinical research and data collection was completed at the research centres of Drs. J.A. Lieberman, H.Y. Meltzer, P. Cavazzoni, and S. Sevy
2.0 Genetic Variation of 5-HT2A receptor and response to clozapine

SIR—Arranz and colleagues (July 29, 1995, p. 281) show that, for the thymidine to cytosine (T to C) nucleotide polymorphism at position 102 of the 5-hydroxytryptamine (serotonin) type 2 receptor (5-HT2A) gene, the genotypes T102/C102 and T102/T102 are more common in schizophrenia patients who respond to clozapine compared to those who do not respond. Clozapine has shown efficacy in 30-60% of patients who have not responded to traditional antipsychotic drugs, and it may induce fewer undesirable extra-pyramidal effects (Meltzer, 1994). This dibenzodiazepine derivative has high affinity for 5-HT2A receptors (Meltzer, 1994), and this may be important for predicting response to the drug. Such prediction is clinically important: responders can be spared delay resulting from failure to respond to other medications and non-responders may be protected from the potential adverse effects of clozapine (e.g., agranulocytosis). The mechanism of antipsychotic action may also be elucidated through investigation of the genetic variants associated with response.

Data from patients with DSM-III-R diagnoses of schizophrenia were collected at the following research clinics: Long Island (JAL), Cleveland (HYM), the Bronx (SS), and Ottawa (PC). After informed consent was obtained, the patients underwent a washout period of 2-4 weeks during which, unless clinically necessary, they received no medication prior to starting clozapine. Treatment continued for a minimum of 6 months before their response to clozapine was determined. This response was defined as a reduction of at least 20% in the Brief Psychiatric Rating Scale (BPRS) from baseline score at enrolment into the study. Clozapine blood levels were monitored during treatment to assess compliance. All genotyping of the patients' DNA, from participating clinics, was
performed at the Clarke Institute of Psychiatry, Toronto. 126 patients were evaluated; 37 women, 89 men (25 African-Americans, 101 whites); mean age was 32.6 (SD 8.4) years. 72 patients responded to clozapine, and 54 did not. Allele frequencies did not differ significantly between patients from different centres or between African-Americans and whites. Age, sex and ethnicity factors were similar between responders and non-responders. Our study yielded no evidence that the genotype or allele frequency of the T→C 102 polymorphism of 5-HT2A is associated with response to clozapine (Table, chi-square test, p=0.5). Our genotypes did not deviate significantly from Hardy-Weinberg equilibrium.

Our failure to replicate Arranz and colleagues' finding may be attributed to sample differences, or to differences in psychiatric rating scales. Our scale (BPRS) is more focused on the measurement of psychopathology than the GAS scale used by Arranz et al. The two studies did not differ in the percentile proportion of responders. There was a difference in the sex ratio. We found no differences in the allele frequencies between the two studies; we therefore combined both data sets (n=275) and found that, overall, responders were more likely to have the genotype T102/C102 than non-responders, and non-responders were more likely to be C102 homozygotes (p=0.014). The allele frequency differences for responders versus non-responders did not reach statistical significance (p=0.055). The T→C 102 polymorphism of 5-HT2A does not alter the predicted amino acid sequence of the receptor, but may be in linkage disequilibrium with a nearby polymorphism which is involved in clozapine response. Further studies are required to assess whether this polymorphism may have a predictive role for response to clozapine that is clinically relevant.
The contributions of Phil Cola, Alfreda Howard, Barry Jones, and Serge Sevy are gratefully acknowledged.

Table: Genotype distributions of the 5-HT2A gene in clozapine-treated patients

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>T102/T102</th>
<th>T102/C102</th>
<th>C102/C102</th>
<th>C102 allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders (n=72)</td>
<td>13 (18%)</td>
<td>40 (56%)</td>
<td>19 (26%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Non-responders (n=54)</td>
<td>6 (11%)</td>
<td>31 (57%)</td>
<td>17 (32%)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

2.1 Statement of significance

This work represents our initial study of genetic variation in the 5-HT2A receptor and clinical response to clozapine, and suggested that a more comprehensive examination of the 5-HT2A receptor gene was warranted. This stimulated the work presented in Chapter 3.
3.0 Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients


As submitted to: Neuropsychopharmacology

Mario Masellis performed the genotyping of the patient sample, the genetic data analysis, and the writing of the manuscript. All clinical research and data collection was performed under the supervision of Drs. H.Y. Meltzer, J.A. Lieberman, and S. Sevy. Mr. P. Cola, and Ms. A. Howard assisted in the collection and assembly of this data at the clinical sites.
3.1 Abstract

Using a pharmacogenetic approach in 185 schizophrenics who have been prospectively assessed for clozapine response, we have examined the hypothesis that polymorphisms in the 5-HT2A (HTR2A), and 5-HT2C (HTR2C) genes are involved in its variable response. A -1437 A→G polymorphism in the putative promoter, and a silent T→C 102 substitution in HTR2A were in almost complete linkage disequilibrium, and neither was associated with response (T→C 102 allele: $\chi^2=0.02$, 1 df, $p=0.90$; genotype: $\chi^2=0.02$, 2 df, $p=0.99$). A his452tyr HTR2A polymorphism was found to be significantly associated with clozapine response (his452tyr allele: $\chi^2=6.43$, 1 df, $p=0.01$; genotype: $\chi^2=6.54$, 2 df, $p=0.04$). No HTR2A haplotype was associated with response. Inter-ethnic differences were observed in the frequencies of the cys23ser HTR2C polymorphism. This polymorphism was not significantly associated with response in either of the ethnic groups (Caucasian and African-American genotype: $\chi^2=3.46$, 2 df, $p=0.18$; $\chi^2=0.31$, 2 df, $p=0.86$, respectively). Although replication is required, the overall results suggest that the his452tyr HTR2A polymorphism may be involved in clozapine response.

**Key Words:** clozapine response - pharmacogenetic - 5-HT2A - 5-HT2C - receptor - polymorphism
3.2 Introduction

Clozapine, the prototype of atypical antipsychotic drugs, has been reported to have a variety of clinical advantages for the treatment of schizophrenia compared to typical antipsychotic drugs. These include: 1) efficacy in treatment-refractory schizophrenia; 2) improvement in negative symptoms; 3) improvement in some types of cognitive function; 4) almost complete absence of extrapyramidal side effects (EPS); 5) virtually no ability to cause tardive dyskinesia (TD) after chronic use; and 6) no increase in serum prolactin levels (reviewed by Meltzer, 1995a). As with all antipsychotic agents, the response to clozapine ranges from none to extensive. Up to 60% of patients refractory or intolerant to typical antipsychotic therapy are significantly improved by clozapine (reviewed by Bleethen, 1993).

This observed variability in response has stimulated interest in identifying predictors of response to clozapine (reviewed by Meltzer, 1996). Identification of one or several variables that can reliably predict clozapine's response could influence the decision on whom to initiate treatment and the duration of a clinical trial. Clinical predictors of good response to clozapine included diagnosis of paranoid schizophrenia, later age of onset, greater number of previous hospitalizations, weight gain, plasma levels of clozapine, and neuroleptic intolerance. On the other hand, female gender, and increased prefrontal cortical sulcal widening have been implicated as clinical predictors of poor response to clozapine. Neurochemical predictors of good response such as low plasma levels of homovanillic acid (HVA), and low cerebrospinal HVA/5-HIAA (5-hydroxyindoleacetic acid) ratios have also been identified. Many of these studies have not yet been replicated independently, and the proportion of the variance observed in response
to clozapine explained by these measures is unknown. For a comprehensive review see Meltzer (1996).

There is strong evidence for a threshold for plasma clozapine levels to achieve good response. It was found that plasma concentrations should equal or exceed 350-400 ng/mL to achieve a high rate of response (see Lindenmayer and Apergi, 1996 for review). Several studies have demonstrated that there is a great deal of inter-individual variability in clozapine's pharmacokinetics (Dahl et al., 1994; Choc et al., 1990; Thorup and Fog, 1977; Bondesson and Lindstrom, 1988; Cheng et al., 1988). Steady-state plasma concentrations of clozapine vary widely between patients treated with the same oral dose. These results suggest that pharmacokinetic factors may play a role in the observed inter-individual variability in response to clozapine.

Traditionally, pharmacogenetic factors based on genetic variation in enzymes that metabolize a drug account for much of the variability in the response and/or side effect profile of that drug (Kalow, 1986). This paradigm has been able to account for variability in response and side effects to several classes of drugs, including typical antipsychotics and antidepressants. Several of these psychotropic agents are metabolized by the polymorphic cytochrome-P450 2D6 (CYP2D6) enzyme (as reviewed by Cholerton et al., 1992, and Dahl and Bertilsson, 1993). This same paradigm may also be applicable to clozapine as preliminary evidence suggests that genetic factors may be involved in the inter-individual variability observed in its response. A recent study has identified monozygotic twins that were concordant for both neuroleptic-resistance and response to clozapine (Vojvoda et al., 1996).
Fischer et al. (1992) reported that clozapine was metabolized by the polymorphic CYP2D6 enzyme and suggested that this might account for the observed inter-individual variability in clozapine's pharmacokinetics. Subsequent studies were unable to confirm this finding (Dahl et al., 1994; Pirmohamed et al., 1995; Jerling et al., 1994; Arranz et al., 1995b). On the other hand, there has been consistent evidence that the hepatic cytochrome P450 isoenzyme, CYP1A2, plays a significant role in the metabolism of clozapine (Jerling et al., 1994; Bertilsson et al., 1994; Odom-White and De Leon, 1996). However, to date, no polymorphisms at this locus have been found (Nakajima et al., 1994). In light of these studies and the lack of polymorphic variation at CYP1A2, it is unlikely that response to clozapine can be predicted using only the traditional pharmacogenetic paradigm of genetic variability in major drug metabolizing enzymes. Nevertheless, this possibility may not be fully excluded without further study. It is also possible that polymorphisms in genes that encode regulators of CYP1A2 expression may be involved.

Central pharmacodynamic factors are likely determinants of inter-individual variation in response to clozapine. Transport across the blood-brain barrier may be involved as well. Several groups have suggested that genetic factors may contribute to differences in pharmacodynamic response to clozapine (Kennedy, 1994; Kerwin et al., 1994; Propping and Nöthen, 1995). Genetic variation in genes that encode receptor proteins for which clozapine has affinity, genes which modify the metabolism of those neurotransmitters, e.g. serotonin and dopamine, and genes which regulate structural proteins which may be indirectly involved in response to clozapine, e.g. serotonin and dopamine transporters, could account for variation in response.
In comparison to typical antipsychotic medications, clozapine has a high affinity for receptors from many different neurotransmitter systems. The cloning of the dopamine D4 receptor and the discovery that clozapine was approximately 10-fold more selective for the D4 receptor than for the D2 receptor (Van Tol et al., 1991) made it a good initial candidate gene to test in examining the pharmacogenetics of clozapine. Several studies of genetic variability in the D4 receptor gene (DRD4) and response to clozapine have produced negative findings (Shaikh et al., 1993; Rao et al., 1994; Shaikh et al., 1995; Rietschel et al., 1996; Badri et al., 1996; Kohn et al., 1997).

There is considerable interest in the role of serotonin receptors in the mechanism of action of clozapine (as reviewed by Meltzer and Nash, 1991). In particular, two of the serotonin 2 receptor subtypes, namely 5-HT2A and 5-HT2C, that clozapine antagonizes with high affinity, have been implicated. 5-HT2A/2C agonists possess potent hallucinogenic properties in humans which are highly correlated with their affinities for both receptors (Glennon et al., 1984), thereby suggesting that these receptors may play a role in psychosis, even though visual hallucinations are rare in schizophrenia. Meltzer and colleagues have hypothesized that some of the clinical advantages of clozapine and other atypical antipsychotics, especially those related to EPS and negative symptoms, may be due to their significantly higher affinity for 5-HT2A receptors relative to their affinity for D2 receptors (Meltzer et al., 1989a; Meltzer and Nash, 1991; Meltzer, 1994; 1995a). 5-HT2C receptors have also been implicated (Canton et al., 1990; Kahn et al., 1993). Furthermore, 5-HT2A/2C antagonists, such as ritanserin and mianserin, may be useful when added to typical antipsychotic therapy. Ritanserin reduces EPS induced by typical antipsychotic drugs
(Bersani et al., 1986) and may even possess antipsychotic properties (Wiesel et al., 1994); mianserin decreases negative symptomatology when added to treatment with typical antipsychotics (Rogue and Rogue, 1992). These findings support a role for 5-HT2A/2C receptor antagonism in the treatment of schizophrenia.

The localization of 5-HT2A and 5-HT2C receptors in the central nervous system is consistent with neuroanatomical structures believed to be involved in the pathophysiology of schizophrenia. 5-HT2A receptors are located on pyramidal neurons in many areas of the frontal cortex as well as some parts of the limbic system, particularly the olfactory nuclei, and parts of the basal ganglia. 5-HT2C receptors, although predominantly found in the choroid plexi, are also distributed in areas of the limbic system, and regions associated with motor behaviour (reviewed by Hoyer et al., 1994).

Recently, Arranz et al. (1995a) have reported an allelic association between a thymidine to cytosine (T→C) silent polymorphism within the coding region of the 5-HT2A receptor gene (HTR2A), located on chromosome 13, and clinical response to clozapine in patients suffering from schizophrenia. They found that the genotype C102/C102 was more common in patients who did not respond to clozapine than in those who did respond. This finding was not replicated by three independent groups (Masellis et al., 1995; Nöthen et al., 1995; Malhotra et al., 1996a). In addition, Nöthen et al. (1995), and Malhotra et al. (1996a) have reported a lack of allelic association between a putative functional polymorphism in HTR2A and clozapine response. This polymorphism causes an amino acid substitution of histidine to tyrosine at position 452 (his452tyr) in the 5-HT2A receptor protein.
An allelic association was also reported between a polymorphism in the 5-HT2C receptor gene (HTR2C), located on the X chromosome, and clozapine response (Sodhi et al., 1995). This polymorphism causes a cysteine to serine amino acid substitution at position 23 (cys23ser) in the 5-HT2C receptor protein. Sodhi et al. (1995) found that the presence of at least one ser23 allele was more common in patients who responded to clozapine than in those who did not. Malhotra et al. (1996b) and Rietschel et al. (1997) were not able to replicate this finding.

These studies were limited in that they were retrospective, medication compliance was not ascertained and, in some cases, they lacked statistical power as a result of a small sample size. We have employed a genetic association strategy to assess four polymorphisms (three in the serotonin 2A receptor gene and one in the serotonin 2C receptor gene) in a large, well-characterized sample of clozapine-treated schizophrenia patients who were prospectively assessed for response to this atypical antipsychotic. The roles of the his452tyr HTR2A polymorphism, a novel adenosine to guanosine (A→G) polymorphism at position -1437 in the putative promoter region of HTR2A, and the cys23ser HTR2C variation were analyzed to determine their contribution in predicting response to clozapine. In addition, we have extended our previous study of the T→C 102 HTR2A polymorphism in prediction of clozapine response.
3.3 Materials and Methods

3.3.A Clinical Sample

Clinical data from patients with DSM-III-R diagnoses of schizophrenia and meeting the criteria for treatment-refractoriness or intolerance to typical antipsychotic therapy (Kane et al., 1988) were obtained at the following research clinics: Case Western Reserve University in Cleveland (HY Meltzer, n=105); Hillside Hospital in Long Island (JA Lieberman, n=65); and Bronx VA (Serge Sevy, n=16). See Table 1 for the demographic distribution of the patients from each of the clinical sites. After informed consent was obtained, the patients underwent a washout period of 2-4 weeks during which, unless clinically necessary, they received no medications prior to starting clozapine. Clozapine treatment was continued for a minimum of six months during which patients were followed prospectively. Clozapine blood levels were also monitored throughout the course of the treatment to ascertain compliance.

Treatment response was evaluated at six months or more using criteria based on those of Kane et al. (1988) in their study of the efficacy of clozapine vs. chlorpromazine in treatment-refractory schizophrenia: a reduction of ≥ 20% in the Brief Psychiatric Rating Scale (BPRS) score from baseline score at enrolment into the study. In cases where a patient was very close to the operational criteria for response (≥15% but <20%) but were clinically much improved, a reduction of at least one category on the Clinical Global Impressions (CGI) scale was considered in order to augment the definition of response; 8 of the 185 patients were classified as responders using this additional criterion. This definition of response was set a priori.
3.3. B Laboratory Methods

Blood samples were collected from the clinical sites and sent to the Clarke Institute of Psychiatry in Toronto. Genomic DNA was extracted from white blood cells using the high-salt method (Lahiri and Nurnberger, 1991). All genotyping of the patients' DNA was performed at the Clarke Institute of Psychiatry and laboratory staff were blind to the psychiatric ratings.

Three polymorphisms in HTR2A, one silent, one that alters the amino acid sequence, and one in its putative promoter region, as well as one putative functional polymorphism in HTR2C were examined in the clinical samples using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique.

The silent T→C 102 polymorphism at the HTR2A locus was genotyped employing a modified protocol of Warren et al. (1993). Primer sequences were the same as those described by Warren et al. (1993). The PCR reaction was performed using 150 ng of genomic DNA, 2.0 mM MgCl2, 0.4 μM of both forward and reverse primer, 200 μM each of 2'-deoxyadenosine-5'-triphosphate (dATP), 2'-deoxycytosine-5'-triphosphate (dCTP), 2'-deoxyguanosine-5'-triphosphate (dGTP), and 2'-deoxythymidine-5'-triphosphate (dTTP), and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 30 cycles of 94°C for 30 seconds, 61°C for 1 minute, and 72°C for 30 seconds with a final extension period of 72°C for 5 minutes using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. The PCR products were then restriction digested using MspI following manufacturer's protocol (New England Biolabs).

The his452tyr HTR2A PCR-RFLP was genotyped using a modified protocol of Erdmann et al. (1996a). Primer sequences were the same as those described by Erdmann
et al. (1996a). The PCR reaction was performed using 150 ng of genomic DNA, 1 mM MgCl₂, 0.6 μM of both forward and reverse primer, 160 μM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds with a final extension period of 72°C for 5 minutes using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. The PCR product was then digested with BbvI restriction endonuclease (New England Biolabs).

The details of the HTR2A -1437 A→G promoter polymorphism were given to us by MM Nöthen (Nöthen et al., 1996). The sequence of the forward primer Pro2F is 5'-cta gcc acc ctg agc cta tg-3'; the sequence of the reverse primer Pro2R is 5'-ttg tgc aga ttc cca tta agg-3'. The PCR reaction was performed using 150 ng of genomic DNA, 1 mM MgCl₂, 0.6 μM of both forward and reverse primer, 160 μM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 30 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. The PCR product was then digested with MspI restriction endonuclease (New England Biolabs). After restriction digestion, the products were separated on a 3% agarose gel by electrophoresis which was stained with ethidium bromide for UV visualization. An A at position -1437 leads to an uncut fragment of 200 base pairs (bp). A G at position -1437 leads to two fragments of length 121 bp and 79 bp.

The cys23ser HTR2C PCR-RFLP was genotyped employing a modified protocol of Lappalainen et al. (1995). The primer sequences were the same as those described by Lappalainen et al. (1995). The PCR reaction was performed using 150 ng of genomic
DNA, 1.5 mM MgCl₂, 0.6 μM of both forward and reverse primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. Restriction digestion was performed using HinfI (New England Biolabs).

All PCR products were separated by agarose gel electrophoresis (2% agarose - T→C 102 HTR2A; 3.5% agarose - his452tyr HTR2A and cys23ser HTR2C) and stained with ethidium bromide for UV visualization.

3.3. C Statistical Methods

The categorical data were analyzed using chi-square tests while the continuous data, e.g. age, have been analyzed using analysis of variance (ANOVA). The statistical program used was the Statistical Package for the Social Sciences (SPSS), version 7.0. Linkage disequilibrium was assessed using the linkage utility program, Equilibrium Haplotype (EH) (Terwilliger and Ott, 1994), which employs a maximum likelihood method. Power analysis was performed using Epi Info, Version 5.01a (Public Domain Software for Epidemiology and Disease Surveillance, March 1991).
3.4 **Results**

There were no significant differences in ethnicity and response rate between the patients from the three clinical sites. There was a significant difference observed between the mean age of the patients from the three clinical sites \( F(2, 181)=5.06, p=0.007 \); using Tukey's HSD test for post hoc comparisons, the mean ages of the Meltzer and Lieberman samples were significantly lower than that of the Sevy sample (see Table 1). There was also a significant difference in the proportion of males to females between the smaller sample (SS) and the two larger samples (HYM and JAL - see Table 1). The smaller sample consisted only of males \( (\chi^2=8.09, 2 \text{ df}, p=0.02, \text{two-tailed}) \). The samples were combined into one group and a total of 185 patients was evaluated: 132 men, 53 women; 144 Caucasians, 40 African-Americans, 1 Asian; the mean age was 33.7 (SD 8.9) years. Of these, 97 (52.4%) patients were considered responders to clozapine by the criteria cited above, and 88 (47.6%) were not. In this combined sample, age, sex and ethnicity factors were not different between responders and non-responders to clozapine.

Genotype frequencies for the three polymorphisms in HTR2A did not differ significantly between patients from the different centres, between patients from different ethnic backgrounds, or between males and females. Genotype frequencies of the HTR2C polymorphism also did not differ significantly between patients from the different centres (hemizygous males and homozygous females were combined - for explanation see below). However, there was a significant difference between genotype frequencies of the HTR2C polymorphism and ethnic status \( (\chi^2=16.87, 2 \text{ df}, p<0.0005 \text{ (two-tailed)} \) - see Table 3); African-Americans had a significantly higher occurrence of the ser23 allele than Caucasians.
For the silent T→C 102 polymorphism in HTR2A, there were no significant differences observed in both allele and genotype counts between responders and non-responders to clozapine [allele: $\chi^2=0.02$, 1 df, $p=0.90$ (two-tailed); genotype: $\chi^2=0.02$, 2 df, $p=0.99$ (two-tailed) - see Table 2a]. We tested for Hardy-Weinberg equilibrium in both the responder and non-responder groups and found no deviation for the T→C 102 polymorphism in HTR2A. The T→C 102 allele frequencies in the total sample were also similar to those reported by other groups. The novel -1437 A→G HTR2A promoter polymorphism was in nearly full linkage disequilibrium with the T→C 102 polymorphism ($\chi^2=305.79$, 1 df, $p<5 \times 10^{-7}$). The disequilibrium coefficient $\delta$ for the haplotype -1437G--C102 was 0.24. That is, almost every patient with a C102 allele had a -1437G allele nearby on the same chromosome. This promoter polymorphism thus measures virtually the same variation as T→C 102 and accordingly the results with respect to clozapine response were similar (results not shown).

There were significant differences observed between responders and non-responders to clozapine for the his452tyr polymorphism in HTR2A (see Table 2b). Allele counts for this polymorphism were significantly different between responders and non-responders to clozapine ($\chi^2=6.43$, 1 df, $p=0.01$, two-tailed, ODDS RATIO for tyr452 association with non-response was 2.42 [95% CI 1.15-5.32]). Patients who failed to respond had a tyr452 allele frequency of 0.15 as compared to 0.07 in those who did respond. Examining genotypes, individuals who did not respond to clozapine were more likely to be his/tyr 452 heterozygotes or tyr/tyr 452 homozygotes than those who did respond ($\chi^2=6.54$, 2 df, $p=0.04$, two-tailed). For this polymorphism, there was no
deviation from Hardy-Weinberg equilibrium in both the responder and non-responder groups. The allele frequencies in the total sample were also comparable to those published by other groups.

The level of linkage disequilibrium between these three HTR2A polymorphisms was high and statistically significant ($\chi^2=305.90, 4 \text{ df}, p<5 \times 10^{-7}$). The disequilibrium coefficient $\delta$ for the haplotype -1437G--C102--his452 was 0.21. Examining only two loci at a time, the main contribution came from the high level of disequilibrium between T→C 102 and -1437 A→G; that between T→C 102 and his452tyr was low and not statistically significant ($\chi^2=0.01, 1 \text{ df}, p=0.92$). In order to determine if a particular haplotype differentiated clozapine responders from non-responders, we performed a haplotype analysis of these three HTR2A markers and response to clozapine. Under both dominant and recessive models, no haplotype association with response was found (dominant model: $p=0.32$; recessive model: $p=0.28$).

Since there was a significant difference between genotype frequencies of the HTR2C polymorphism and ethnic status, we analyzed clinical response to clozapine in Caucasians and African-Americans separately. Since HTR2C is X-linked, hemizygous males and homozygous females were grouped together due to inactivation of one of the X chromosomes in females. When the data were analyzed in this fashion (see Table 3), there were no significant differences observed between Caucasian responders and non-responders to clozapine ($\chi^2=3.46, 2 \text{ df}, p=0.18$, two-tailed). This was also the same in the group of African-Americans when clozapine response was considered ($\chi^2=0.31, 2 \text{ df}, p=0.86$, two-tailed). However, examination of the genotype distribution for both ethnic
groups revealed a tendency for responders to be hemizygous or homozygous for the ser23 variant.

**Table 1:** Demographic distribution from each of the clinical sites

<table>
<thead>
<tr>
<th></th>
<th>Meltzer (n=105)</th>
<th>Lieberman (n=64)</th>
<th>Sevy (n=16)</th>
<th>Total (n=185)</th>
</tr>
</thead>
</table>
| **Age**  
(Mean±SD)        | 32.5±8.9        | 34.0±8.1         | 40.1±9.4    | 33.7±8.9     |
| **Sex**         |                 |                  |             |              |
| Male            | 75              | 41               | 16          | 132 (71.4%)  |
| Female          | 30              | 23               | 0           | 53 (28.6%)   |
| **Ethnicity**   |                 |                  |             |              |
| Caucasian       | 79              | 53               | 12          | 144 (77.9%)  |
| Afro-American   | 26              | 10               | 4           | 40 (21.6%)   |
| Asian           | 0               | 1                | 0           | 1 (0.5%)     |
| **Response**    |                 |                  |             |              |
| Responders      | 51              | 37               | 9           | 97 (52.4%)   |
| Non-responders  | 54              | 27               | 7           | 88 (47.6%)   |

* F(2, 181)=5.06, p=0.007 for age vs. clinical site.

*χ^2^=8.09, 2 df, p=0.02 (two-tailed) for sex vs. clinical site.
Table 2a: Allele and genotype counts and frequencies of the T→C 102 polymorphism in the 5-HT2A receptor gene in clozapine-treated patients

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T102</td>
<td>86 (45%)</td>
<td>79 (46%)</td>
<td>165 (46%)</td>
</tr>
<tr>
<td>C102</td>
<td>104 (55%)</td>
<td>93 (54%)</td>
<td>197 (54%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>190</td>
<td>172</td>
<td>362</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T102/T102</td>
<td>19 (20%)</td>
<td>18 (21%)</td>
<td>37 (21%)</td>
</tr>
<tr>
<td>T102/C102</td>
<td>48 (51%)</td>
<td>43 (50%)</td>
<td>91 (50%)</td>
</tr>
<tr>
<td>C102/C102</td>
<td>28 (29%)</td>
<td>25 (29%)</td>
<td>53 (29%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>95</td>
<td>86</td>
<td>181</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.02, \text{ 1 df, } p=0.90 \text{ (two-tailed) for clinical response relative to allele counts \{ODDS RATIO for C102 association to non-response}=0.97; 95\% \text{ CI 0.63-1.50}}. \]

\[ \chi^2 = 0.02, \text{ 2 df, } p=0.99 \text{ (two-tailed) for clinical response relative to genotype counts.} \]
Table 2b: Allele and genotype counts and frequencies of the his452tyr polymorphism in the 5-HT2A receptor gene in clozapine-treated patients

<table>
<thead>
<tr>
<th>Allele</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>His452</td>
<td>177 (93%)</td>
<td>146 (85%)</td>
<td>323 (89%)</td>
</tr>
<tr>
<td>Tyr452</td>
<td>13 (7%)</td>
<td>26 (15%)</td>
<td>39 (11%)</td>
</tr>
<tr>
<td>Total</td>
<td>190</td>
<td>172</td>
<td>362</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>His/His 452</td>
<td>82 (86%)</td>
<td>63 (73%)</td>
<td>145 (80%)</td>
</tr>
<tr>
<td>His/Tyr 452</td>
<td>13 (14%)</td>
<td>20 (23%)</td>
<td>33 (18%)</td>
</tr>
<tr>
<td>Tyr/Tyr 452</td>
<td>0 (0%)</td>
<td>3 (4%)</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>86</td>
<td>181</td>
</tr>
</tbody>
</table>

*χ²=6.43, 1 df, p=0.01 (two-tailed) for clinical response relative to allele counts [ODDS RATIO for tyr452 association to non-response=2.42; 95% CI 1.15-5.32].  
*χ²=6.54, 2 df, p=0.04 (two-tailed) for clinical response relative to genotype counts.
Table 3: Genotype counts and frequencies of the cys23ser polymorphism in the 5-HT2C receptor gene in Caucasian and Afro-American clozapine-treated patients

<table>
<thead>
<tr>
<th>Genotype in</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caucasians</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys or Cys/Cys 23</td>
<td>55 (76%)</td>
<td>58 (87%)</td>
<td>113 (81%)</td>
</tr>
<tr>
<td>Cys/Ser 23</td>
<td>4 (6%)</td>
<td>4 (6%)</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>Ser or Ser/Ser 23</td>
<td>13 (18%)</td>
<td>5 (7%)</td>
<td>18 (13%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>72</td>
<td>67</td>
<td>139</td>
</tr>
</tbody>
</table>

| Genotype in       |            |                |       |
| **Afro-Americans**|            |                |       |
| Cys or Cys/Cys 23 | 9 (45%)    | 10 (53%)       | 19 (49%) |
| Cys/Ser 23        | 3 (15%)    | 3 (16%)        | 6 (15%) |
| Ser or Ser/Ser 23 | 8 (40%)    | 6 (31%)        | 14 (36%) |
| **Total**         | 20         | 19             | 39    |

\(^a\) \chi^2=3.46, 2 df, p=0.18 (two-tailed) for clinical response relative to genotype counts in Caucasians.

\(^b\) \chi^2=0.31, 2 df, p=0.86 (two-tailed) for clinical response relative to genotype counts in Afro-Americans.

\(^c\) \chi^2=16.87, 2 df, p<0.0005 (two-tailed) for genotype counts of Caucasians relative to Afro-Americans (Asian individual excluded from the analysis). N.B. Hemizygous males and homozygous females grouped together because HTR2C is X-linked.
3.5 Discussion

We have previously reported the lack of allelic association between the T→C 102 polymorphism in HTR2A and clozapine response in a sample of clozapine-treated patients (Masellis et al., 1995). In this current study, we have examined this polymorphism in an extended sample and, once again, report a lack of allelic association. Thus, we were not able to replicate the results of Arranz et al. (1995a) who found that the homozygous genotype C102/C102 was more frequent in the non-responders than in the responders to clozapine. Two other groups (Nothen et al., 1995; Malhotra et al., 1996a) have also failed to replicate the finding of Arranz et al. (1995a).

The T→C 102 HTR2A polymorphism does not alter the predicted amino acid sequence of the receptor. One explanation for the discrepancy between our results and those of Arranz et al. (1995a) might be that this silent HTR2A polymorphism is in linkage disequilibrium with a nearby polymorphism which is involved in clozapine response in the Arranz et al. sample but not in our sample. This might be due to differences in the populations from which the samples were collected. For example, the Arranz et al. sample consisted of Caucasian schizophrenia patients of Western European origin, whereas our sample consisted of North American schizophrenia patients from a greater variety of ethnic backgrounds.

Our failure to replicate Arranz and colleagues’ finding may also be attributed to differences in the psychiatric rating scales used. We employed the Brief Psychiatric Rating Scale (BPRS) which is more focused on psychopathology than the Global Assessment Scale (GAS) used by Arranz et al. (1995a). The GAS includes an assessment of social functioning in its ratings and this may be only loosely linked with psychopathology. The
patients from the largest site (n=105; HYM) in this study were also assessed with the GAS in addition to the BPRS. It was, therefore, possible in this group to employ the same criteria used by Arranz et al. (1995a) to define response, that is, a 20 point improvement in GAS score following clozapine treatment. According to the GAS criteria of Arranz et al., 18% of our patients (89 individuals - 16 individuals had GAS data missing) were responders to clozapine, whereas 48.6% (105 individuals - total sample) were responders using our definition of response. Therefore, a smaller proportion of this total patient sample (HYM) responded as well to clozapine using the Arranz et al. GAS definition of response. The Arranz study utilized retrospective ratings only and may have overestimated the extent of change for that reason. Although we do not have access to the GAS scores for the other two samples (JAL, SS), these patients were clinically similar to the Meltzer sample. It may be appropriate to use multiple criteria for assessing outcome because the genetic influence on outcome may be most important for only some of the relevant outcome measures, e.g. EPS, negative symptoms, and quality of life. A standard definition of antipsychotic response which includes multiple criteria should be agreed upon for use in future pharmacogenetic studies to attempt to control for this potential confounding factor.

We have found evidence for an association between the polymorphism that codes for a histidine to tyrosine amino acid substitution at position 452 in the 5-HT2A receptor protein. We found that non-responders to clozapine were more likely to possess the rarer tyr452 variant than those who responded to clozapine. This amino acid substitution occurs in the intracellular carboxyl-terminal tail of the 5-HT2A receptor protein. A substitution of a basic histidine residue for an uncharged polar tyrosine residue in the 5-
HT2A receptor at this position, may result in altered tertiary structure and possibly altered receptor function. The carboxyl-terminal domain of G-protein coupled receptors has been implicated in phosphorylation-dependent desensitization (Hausdorff et al., 1990). Recently, Ozaki et al. (1997) have demonstrated that, in platelets from individuals possessing the different alleles, the tyr452 form of the 5-HT2A receptor protein may exist in a partially desensitized state in comparison to the his452 form. In his/tyr 452 heterozygotes, they found that tyr452 was associated with a smaller peak amplitude of Ca$^{2+}$ mobilization after stimulation with serotonin compared to his452. Tyr452, as opposed to his452, was also found to be associated with a different time course of Ca$^{2+}$ mobilization after stimulation with serotonin. These functional differences may account for the positive association observed here between this structural HTR2A variation and clozapine response. Alternatively, this particular variant may be in linkage disequilibrium with another site in HTR2A conferring response to clozapine. A false positive result is also a possible explanation.

Arranz et al. (1996) have recently reported a similar trend between this his452tyr HTR2A polymorphism and response to clozapine in a sample of Western European schizophrenia patients. The study of Malhotra et al. (1996a) did not support these results. Their failure to replicate the finding of Arranz et al. (1996) and those reported here may be due to differences in the clinical methods of the clozapine trials. First of all, the patient sample of Malhotra et al. was more clinically heterogeneous than ours as their study included clozapine-treated patients with either DSM-III-R diagnoses of schizophrenia or schizoaffective disorder; it was also not clear if they were treatment-refractory or intolerant and their sample size was substantially smaller (n=70). Our study included only
individuals who had DSM-III-R diagnoses of schizophrenia and who were treatment-refractory or intolerant to typical antipsychotic agents. Secondly, Malhotra et al. (1996a) assessed baseline BPRS scores after 4 weeks of clinical stabilization on typical antipsychotic. Our sample of patients went through a washout period of 2 to 4 weeks, at which point baseline BPRS scores were assessed. Therefore, the mean baseline BPRS score of Malhotra et al. (1996a) may be lower than ours. Finally, their patient sample was treated with clozapine for only 10 weeks. All of our patients were maintained on clozapine treatment for a minimum of 6 months.

There is controversy surrounding the issue of the influence of duration of clozapine treatment and change in psychopathology. Meltzer (1989b; 1995b) has observed that some patients treated with clozapine may not reach criteria for response until after six months of treatment. Lieberman et al. (1994) also noted that some patients continue to improve on clozapine for up to a year and has supported the idea that the optimal period for a trial of clozapine is 12 to 24 weeks. On the other hand, Carpenter et al. (1995b) have suggested that clozapine treatment be discontinued if no improvement is observed within several weeks. Approximately 40% of patients respond to clozapine with a 20% decrease in total BPRS score within the first 10 weeks of treatment assuming that: 1.) the dose is increased at a reasonable rate; 2.) the patients are not receiving typical antipsychotics concomitantly; 3.) they are treatment-refractory to begin with; and 4.) have baseline total BPRS scores ≥40. Between 6 and 12 months of treatment, the response rate should approach 60%. Assuming a high response rate of 40% in the first 10 weeks, anywhere from 10 to 20% of patients should subsequently respond to clozapine in the next 14 weeks. Lieberman et al. (1994) has also found that up to 20% of patients may respond
after the initial 10 weeks of treatment. Based on this evidence, the proportion of responders vs. non-responders will differ at 10 and 24 weeks and thus the distribution of alleles in each of these groups may be significantly different from each other. This duration of treatment factor may contribute to the differences observed between our results and those of Malhotra et al. (1996a). In future pharmacogenetic studies, it seems appropriate that groups working in this field should agree upon a standard duration of time for clozapine treatment. If allele frequencies are not rare, then studies which include equal numbers of responders and non-responders may most likely be informative.

Our study is also different from others examining the pharmacogenetics of clozapine response in that our patients were prospectively followed up at regular intervals; the other studies were conducted retrospectively. These previous studies also did not measure blood levels of clozapine such that poor compliance or sub-threshold blood levels may have been factors. The patients in our study had their clozapine blood levels carefully monitored at regular intervals to ascertain compliance and to ensure that the plasma level threshold was achieved.

There was no association observed between any of the particular haplotype combinations of the HTR2A markers and response to clozapine. To our knowledge, this is the first haplotype analysis of HTR2A performed in a clozapine response sample looking specifically for a relationship between particular combinations of alleles and response to clozapine. Our results indicate that the only polymorphism contributing significantly to the prediction of clozapine response was the his452tyr HTR2A structural polymorphism. No particular haplotype was associated with clozapine response under both dominant and recessive models.
We were not able to replicate the finding of Sodhi et al. (1995), in which they found a positive association between the cys23ser amino acid substitution in the 5-HT2C receptor protein. Malhotra et al. (1996b) and Rietschel et al. (1997) also were not able to replicate this finding. Our failure to replicate may be due to the differences previously discussed between our study and that of Arranz et al. (1995a). Although we have failed to find a positive result between HTR2C and clozapine response, there was a tendency for responders to be either hemizygous or homozygous for the ser23 variant and this was in the same direction as that reported by Sodhi et al. (1995). Further investigation of this variant is warranted as Goldman et al. (1995) have preliminary evidence suggesting that the ser23 HTR2C variant has altered affinity for the serotonergic agonist meta-chlorophenylpiperazine (mCPP) when compared to the cys23 variant. It is possible that substitution of a cys23 residue with a ser23 residue alters the tertiary structure of the 5-HT2C receptor protein by disrupting formation of protein-stabilizing disulfide bonds between cysteine residues; this may lead to altered receptor function.

To our knowledge, our study is the first to report ethnic differences between Caucasians and African-Americans in the genotypic distribution of the cys23ser HTR2C polymorphism. Although replication in larger samples are needed to confirm this initial finding, this may be relevant to future studies examining differences in antipsychotic response across ethnic groups.

One of the most important limitations in studies of a complex trait such as clozapine response is sample size. It is difficult to collect large, well-characterized samples such as the one reported here. In addition, response to clozapine is thought to involve interaction with many different receptor sites and is thus likely to be genetically
heterogeneous. Therefore, the ability to detect the effects of genes that play only a minor role in predicting response to clozapine is limited by this lack of statistical power. Allowing a type I error rate ($\alpha$)=0.05, power ($1-\beta$)=0.80, and proportion of the responders with the risk allele for non-response ($P_0$)=0.60, we can detect an odds ratio for the C102 variant as low as 1.92 in our sample of clozapine-treated patients. Specifying the same $\alpha$ and power ($1-\beta$) with a $P_0$=0.11, we are able to detect an odds ratio for the tyr452 variant as low as 2.33 in our sample. Therefore our sample has enough power to identify allelic variants in genes that may contribute in a more minor way to the total variance observed in clozapine response. Another limitation of our study is multiple testing. Replication in independent samples is required to confirm our finding. These samples are currently in the process of being collected.

Recently, Inayama et al. (1996), Erdmann et al. (1996a), and Williams et al. (1996) have reported positive associations between the T→C 102 variant of HTR2A and schizophrenia using traditional case-control association studies. They found that the C102 variant was more common in schizophrenia patients than in controls and may be a gene of minor effect in liability to develop schizophrenia. Several replication studies employing the Haplotype Relative Risk (HRR) strategy, a more powerful method than traditional case-control studies which eliminates biases due to population stratification, have produced negative results (Malhotra et al., 1996c; Verga et al., 1997). Although these HRR findings argue against a role of HTR2A in schizophrenia, a recent meta-analysis of all published HTR2A-schizophrenia association studies (Williams et al., 1997) provides further support of a role for this gene, albeit minor, in contributing to susceptibility to schizophrenia.
Schizophrenia is thought to be a complex, non-Mendelian genetic disease with an uncertain mode of inheritance, incomplete penetrance, and probable genetic heterogeneity (Gottesman, 1991). Thus identifying genes for this disorder has proven to be a difficult task. Focusing in on particular clinical phenotypes or subgroups of schizophrenics, such as responders and non-responders to clozapine, may reduce the overall heterogeneity making it possible to work with a trait that is more Mendelian in nature, and more likely to be homogeneous (Lander, 1988; Lander and Schork, 1994). This hypothetically should make it easier to identify genes for that trait. The results of our current study, employing response subgroups, provides support for a role of HTR2A in clozapine response, which in turn provides additional support for its involvement in schizophrenia.

From these pharmacogenetic studies, one or more genetic factors may be discovered that play an important role in antipsychotic medication response. This genetic information may aid clinicians in deciding who should receive a particular drug, and it may also help in elucidating the mechanism of action of atypical antipsychotic agents. Ultimately, the genetic information may lead to the design of new, more specific therapeutic agents, and may illuminate our understanding of the pathophysiology of schizophrenia.
3.6 Results not shown

Table 4: Allele and genotype counts and frequencies of the -1437 A→G polymorphism in the 5-HT2A receptor gene in clozapine-treated patients

<table>
<thead>
<tr>
<th>Allele</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1437A</td>
<td>81 (47%)</td>
<td>80 (49%)</td>
<td>161 (48%)</td>
</tr>
<tr>
<td>-1437G</td>
<td>91 (53%)</td>
<td>84 (51%)</td>
<td>175 (52%)</td>
</tr>
<tr>
<td>Total</td>
<td>172</td>
<td>164</td>
<td>336</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1437A/A</td>
<td>21 (25%)</td>
<td>21 (26%)</td>
<td>42 (25%)</td>
</tr>
<tr>
<td>-1437A/G</td>
<td>39 (45%)</td>
<td>38 (46%)</td>
<td>77 (46%)</td>
</tr>
<tr>
<td>-1437G/G</td>
<td>26 (30%)</td>
<td>23 (28%)</td>
<td>49 (29%)</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>82</td>
<td>168</td>
</tr>
</tbody>
</table>

$^a \chi^2=0.10, 1 \text{ df}, p=0.76$ (two-tailed) for clinical response relative to allele counts. $^b \chi^2=0.10, 2 \text{ df}, p=0.95$ (two-tailed) for clinical response relative to genotype counts.

3.7 Statement of significance

This work has assessed all of the known genetic variability observed in both the 5-HT2A and 5-HT2C receptors and their role in prediction of response to clozapine. In addition to demonstrating an association between an HTR2A polymorphism and clozapine response, this research has critically reviewed and analyzed the phenotype of clozapine response and several recommendations have been made with respect to future studies.
4.0 Polymorphisms in the serotonin 5-HT1A, 5-HT6, and 5-HT7 receptor genes and prediction of response to clozapine


Mario Masellis performed the genotyping of the patient sample, the genetic data analysis, and the writing of the manuscript. All clinical research and data collection was performed at the research clinics of Drs. H.Y. Meltzer, J.A. Lieberman, and S. Sevy.
4.1 Abstract

The ‘atypical’ pharmacological profile of clozapine is thought to contribute to its many unique clinical attributes in the treatment of schizophrenia. In particular, clozapine’s high affinity for receptors from the serotonergic system has been hypothesized to be involved in its mechanism of action. This hypothesis was examined using a pharmacogenetic approach in a group (n=185) of schizophrenia patients that have been well characterized for response to clozapine. Polymorphisms in the 5-HT1A (HTR1A), 5-HT6 (HTR6), and 5-HT7 (HTR7) receptor genes were genotyped. The pro16leu HTR1A polymorphism was not observed in our sample; all individuals genotyped were pro/pro 16 homozygotes. With respect to the pro279leu HTR7 polymorphism, one Caucasian male responder to clozapine was observed to be heterozygous (pro/leu 279 genotype). This individual was clinically similar to the other clozapine responders. No evidence for either an allelic or genotypic association of the T→C 267 HTR6 polymorphism with response to clozapine was found in our sample (allele: χ²=0.06, 1 df, p=0.80; genotype: χ²=0.06, 1 df, p=0.80).
4.2 Introduction

Response to the atypical antipsychotic, clozapine, is highly variable in treatment-refractory and/or intolerant schizophrenia patients (Bleehen, 1993). The ability to predict response to clozapine would be important from a clinical perspective: predicted responders to this drug could be treated immediately, and non-responders could be spared from the potential adverse effects (e.g. agranulocytosis). However, finding specific predictors of response to clozapine has proven to be a difficult task. Clozapine’s wide spectrum of unique clinical effects for the treatment of schizophrenia (reviewed by Meltzer, 1995a) suggests that many factors may be involved. Clozapine has high affinity for receptors from many different neurotransmitter systems and several of these interactions have been implicated in its mechanism of action (reviewed by Ashby and Wang, 1996). Response to clozapine is thus likely to be a complex phenomenon involving either a simultaneous interaction with multiple receptor sites or interactions with different receptor sites in different clinical subgroups of patients.

Pharmacogenetics seeks to identify polymorphisms or genetic variation in or near the coding region of protein structures with which a drug interacts that are hypothesized to be involved in the observed variability in its clinical profile. This paradigm can be applied to examine the variable responsiveness to clozapine. Recent advances in molecular genetics allow for the identification of genes involved in complex traits (Bennett et al., 1995). These techniques are well suited for examining the trait of clozapine response as there are probably several receptors involved.

Clozapine has high affinity for receptors from the serotonin system (Meltzer, 1994). Previous pharmacogenetic studies of clozapine response and the serotonin system...
have examined the 5-HT2A and 5-HT2C receptor genes (Arranz et al., 1995a; Arranz et al., 1996; Malhotra et al., 1996a; 1996b; Masellis et al., 1995; Masellis et al., submitted; Nöthen et al., 1995; Rietschel et al., 1997; Sodhi et al., 1995). These studies have yielded largely inconsistent results. Apart from the obvious reason that the reported positive results could be false, it is also possible that different genes are operating in different samples of patients. That is, variability in one particular candidate gene contributes to the phenotype of response in some patients while another may be operating in another group of patients. Alternatively, polymorphisms across several candidate loci each contribute to a small amount of the total variance observed in the phenotype of response. These concepts are plausible given the genetic heterogeneity of schizophrenia and the likely involvement of multiple loci contributing to the disorder (McGuffin et al., 1995). The current study has examined candidate genes from the serotonin system, other than HTR2A and HTR2C, that may be involved in the phenotype of clozapine response. A brief rationale for studying the 5-HT1A (HTR1A), 5-HT6 (HTR6), and 5-HT7 (HTR7) receptor genes in clozapine responders and non-responders will now be provided.

5-HT1A agonism has been shown to prevent and ameliorate neuroleptic-induced catalepsy in rodents (Broekkamp et al., 1988; Hicks, 1990; Invernizzi et al., 1988; Neal-Beliveau, 1993; Wadenberg and Ahlhenius, 1991; Wadenberg, 1992). This effect has also been observed in primate models of extrapyramidal symptoms (EPS) (Casey, 1992; Casey, 1994; Liebman et al., 1989). Agonism of 5-HT1A receptors has been shown to lead to a decrease in the firing of raphe-nigral serotonin neurons and this, in turn, disinhibits the dopaminergic nigrostriatal neurons increasing dopamine levels at the striatum (Arborelius et al., 1993; Kelland et al., 1990; Sinton and Fallon, 1988). This mechanism is thought to
be involved in the reduction of catalepsy and EPS as these phenomena are caused by blockade of D2 receptors in the striatum.

Newman-Tancredi et al. (1996) have recently demonstrated that clozapine acts as a partial agonist at 5-HT1A receptors. It is possible that 5-HT1A agonism is involved in the mechanism of action of clozapine, especially with respect to reduced EPS. Furthermore, 5-HT1A receptor sites are localized in many areas of the brain including the hippocampus, the septum, the amygdaloid, the neocortex, the hypothalamus, and the raphe nuclei, particularly the dorsal raphe (reviewed by Hoyer et al., 1994). Many of these areas are components of the limbic system which is primarily responsible for the modulation of emotion. Based on this information, the 5-HT1A receptor gene (HTR1A) is a good candidate to test for clozapine response. HTR1A is located on human chromosome 5q and a cytosine to thymidine (C→T) polymorphism at position 47 of this gene which causes an amino acid substitution of proline to leucine at position 16 of the 5-HT1A receptor protein has been identified in a Japanese sample (Inayama et al., 1995). This polymorphism was genotyped in our group of clozapine-treated patients.

The human 5-HT6 receptor gene (HTR6) has recently been cloned and has been localized to chromosome 1p (Kohen et al., 1996). A silent thymidine to cytosine (T→C) polymorphism at position 267 of the first exon of HTR6 has recently been identified (Kohen et al., 1996) and this has been assessed for association with clozapine response in our sample. mRNA for the 5-HT6 receptor has been found in several human brain regions including the caudate nucleus, the hippocampus, and the amygdala; lower levels were observed in the thalamus, subthalamic nuclei, and the substantia nigra (Kohen et al., 1996). Several of these central nervous system (CNS) areas have been implicated in the
pathophysiology of schizophrenia. 5-HT6 receptors have been shown to be positively coupled to adenylyl cyclase (Sebben et al., 1994; Kohen et al., 1996), however, precise biochemical and behavioural effects have yet to be determined. Clozapine and several other atypical antipsychotic agents are antagonists at and have demonstrated high affinity for 5-HT6 receptors (Glatt et al., 1995; Kohen et al., 1996; Roth et al., 1994). Approximately 40% of clozapine binding sites in the rat brain resemble pharmacologically the 5-HT6 receptor (Glatt et al., 1995), and based on relative affinities of clozapine for D2 and 5-HT2A receptors, 5-HT6 receptors should be highly occupied at clinically relevant doses of clozapine (Kohen et al., 1996). This suggests that 5-HT6 receptors may be important in the mechanism of action of clozapine and other atypical antipsychotic agents.

Clozapine is an antagonist at and possesses high affinity for 5-HT7 receptors (Roth et al., 1994). mRNA and in situ hybridization studies have demonstrated that 5-HT7 receptors may be expressed in the CNS in the hypothalamus, the anteroventral and paraventricular thalamic nuclei, and the hippocampus (reviewed by Eglen et al., 1997). Expression of mRNA in these midline, thalamic, and limbic structures suggest a role of 5-HT7 in the regulation of emotion (Eglen et al., 1997) and some of these structures may be involved in the pathophysiology of schizophrenia. 5-HT7 receptors are positively coupled to adenylyl cyclase (Bard et al., 1993; Ruat et al., 1993), and this receptor subtype may be involved in the regulation of circadian rhythm phase shifts (Meyerhof et al., 1993; Ying and Rusak, 1997). This role of the 5-HT7 receptor may be interesting considering that some schizophrenics experience a reversal of the sleep-wake cycle accompanied by severe insomnia (Benca, 1996). The gene encoding the 5-HT7 receptor has been localized to human chromosome 10q, and a cytosine to thymidine (C→T) polymorphism at position
leading to a proline to leucine amino acid substitution at position 279 in the third intracellular loop of the receptor protein was genotyped in our group of clozapine-treated patients (Pesonen et al., 1995; Erdmann et al., 1996b).

4.3 Methods

4.3A Clinical Sample

Clinical data from patients with DSM-III-R diagnoses of schizophrenia and meeting the criteria for treatment-refractory or intolerant disease (Kane et al., 1988) were obtained at the following research clinics: Case Western Reserve University in Cleveland (HY Meltzer, n=105); Hillside Hospital in Long Island (JA Lieberman, n=65); and Bronx VA (Serge Sevy, n=16). After informed consent was obtained, the patients underwent a washout period of 2-4 weeks during which, unless clinically necessary, they received no medications prior to starting clozapine. Clozapine treatment was continued for a minimum of six months during which patients were followed prospectively. Clozapine blood levels were also monitored throughout the course of the treatment to ascertain compliance.

Treatment response was evaluated at six months or more using criteria based on those of Kane et al. (1988) in their study of the efficacy of clozapine vs. chlorpromazine in treatment-refractory schizophrenia: a reduction of $\geq 20\%$ in the Brief Psychiatric Rating Scale (BPRS) score from baseline score at enrolment into the study. In cases where a patient was very close to the operational criteria for response ($\geq 15\%$ but $< 20\%$) but were clinically much improved, a reduction of at least one category on the Clinical Global
Impressions (CGI) scale may have been considered to augment the definition of response; 8 of the 185 patients were classified as responders using this additional criterion.

4.3.B Laboratory Methods

Blood samples were collected from the clinical sites and sent to the Clarke Institute of Psychiatry in Toronto. Genomic DNA was extracted from white blood cells using the high-salt method (Lahiri and Nurnberger, 1991). All genotyping of the patients' DNA was performed at the Clarke Institute of Psychiatry blind to the psychiatric ratings.

One biallelic polymorphism in each of the three candidate genes, HTR1A, HTR6, and HTR7, was genotyped in our group of clozapine-treated patients. The laboratory techniques will now be described.

The proline polymorphism, resulting from a cytosine to thymidine (C→T) substitution at position 47 of the HTR1A locus, was genotyped employing allele-specific oligonucleotide (ASO) hybridization. The polymerase chain reaction (PCR) was used to amplify a 411 base pair region of HTR1A from position -66 to 345. The forward and reverse PCR primers used were 5'-aag ggg cga ggc gaa tgt ctc cgc tgt-3', and 5'-gag ggc gat gaa cag gc gca ggt tac-3', respectively. Primer sequences were obtained from Inayama et al. (1995). The PCR reaction was performed using 150 ng of genomic DNA, 1.0 mM MgCl₂, 0.6 μM of both forward and reverse primer, 160 μM each of 2'-deoxyadenosine-5’-triphosphate (dATP), 2’-deoxycytosine-5’-triphosphate (dCTP), 2’-deoxyguanosine-5’-triphosphate (dGTP), and 2’-deoxythymidine-5’-triphosphate (dTTP), and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds using a GeneAmp 9600 Perkin-Elmer Cetus PCR
After PCR amplification, 5 μL of the amplification product was diluted in 200 μL of 15×SSC which was then denatured into single-stranded DNA by boiling followed by snap-cooling. This was then applied onto Hybond-N+ membrane by slot-blotting, dividing the product equally between wells in two lanes. The end result was two sets of identical blots. The blots were then UV-crosslinked and were prehybridized in 5×SSPE, 6% PEG, 5×Denhardt’s solution and 0.5% SDS for one hour at 35°C. Allele-specific oligonucleotides (ASOs) were designed with the following sequences pro16 ASO: 5’-cac cac ggc tcc-3’, and leu16 ASO: 5’-cac cac t ggc tcc-3’. These ASOs were end-labelled with γ32P, and the pro16 ASO was hybridized to one set of the identical blots while the leu16 ASO was hybridized to the other (hybridization temperature 35°C; hybridization time: one hour). The blots were washed in 3×SSC/0.1% SDS at 44°C for the leu16 allele, and 46°C for the pro16 allele to remove the non-complementary oligonucleotide. The blots were then subjected to autoradiography to visualize the variants.

The T→C 267 polymorphism at the HTR6 locus was genotyped employing the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique. A modified protocol of Kohen et al. (1996) was used. Primer sequences were as follows: forward, 5’-tgc tga tcg cgc tca tct gca ctc a-3’ and reverse, 5’-ctg cag cgt ctc cga ggc ctg act g-3’. The PCR reaction was performed using 200 ng of genomic DNA, 1.0 mM MgCl2, 0.8 μM of both forward and reverse primer, 200 μM each of dATP, dCTP, 90% dGTP + 10% 7-deaza-2’-deoxyguanosine-5’-triphosphate (deaza-dGTP), and dTTP, 10% dimethyl sulfoxide (DMSO), and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 45
cycles of 95°C for 40 seconds, 60°C for 40 seconds, and 72°C for 40 seconds using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. The PCR products were then restriction digested using Rsal following manufacturer’s protocol (New England Biolabs). The digested products were electrophoresed on 3.5% agarose gels which were stained with ethidium bromide for UV visualization.

The pro279leu polymorphism at the HTR7 locus was genotyped employing the PCR-RFLP technique. Pesonen et al. (1995) first described this polymorphism, and we obtained the specific methodological details from D Goldman. Forward and reverse primer sequences were as follows: 5'-gat tct ctc cgt cgt gct tct-3', and 5'-gca cac tct tcc acc tcc ttc-3', respectively. The PCR reaction was performed using 150 ng of genomic DNA, 1.0 mM MgCl₂, 0.6 μM of both forward and reverse primer, 160 μM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 25 μL. The PCR program consisted of 30 cycles of 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds using a GeneAmp 9600 Perkin-Elmer Cetus PCR machine. The PCR products were then restriction digested using XhoI following manufacturer’s protocol (New England Biolabs). The digested products were electrophoresed on 3.0% agarose gels which were stained with ethidium bromide for UV visualization. XhoI recognizes the pro279 variant producing two fragments of length 230 bp and 72 bp but leaves the leu279 variant uncut (302 bp).

4.3.C Statistical Methods

The categorical data were analyzed using chi-square tests while the continuous data, e.g. age, have been analyzed using ANOVA. The statistical program used was the Statistical Package for the Social Sciences (SPSS), version 7.0. Power analysis was
performed using Epi Info, Version 5.01a (Public Domain Software for Epidemiology and Disease Surveillance, March 1991).

4.4 Results

There were no significant differences in ethnicity and response rate between the patients from the three clinical sites. There was a significant difference observed between the mean age of the patients from the three clinical sites [F(2, 181)=3.43, p=0.007]; using Tukey’s HSD test for post hoc comparisons, the mean ages of the Meltzer and Lieberman samples were significantly lower than that of the Sevy sample (results not shown - refer to Table 1 in Chapter 3). There was also a significant difference in the proportion of males to females between the Sevy sample (SS) and the two larger samples (HYM and JAL; results not shown - see Table 1 in Chapter 3). The smaller sample consisted predominantly of males ($\chi^2=8.09, 2$ df, p=0.02, two-tailed). The samples were combined into one group and a total of 185 patients was evaluated: 132 men, 53 women; 144 Caucasians, 40 African-Americans, 1 Asian; the mean age was 33.7 (SD 8.9) years. Of these, 97 (52.4%) patients were considered responders to clozapine by the criteria cited above, and 88 (47.6%) were not. In this combined sample, age, sex and ethnicity factors were not different between responders and non-responders to clozapine.

The HTR1A pro16leu amino acid substitution was not found to be polymorphic in our sample; all individuals were pro/pro 16 homozygotes. The leu279 variant of the pro279leu HTR7 polymorphism was observed in only one heterozygous individual in the entire patient sample. This patient was a 23 year old Caucasian male and was a responder.
to clozapine. There were no outstanding clinical features that distinguished this person from the rest of the responders.

Genotype frequencies for the T→C 267 HTR6 polymorphism were not significantly different between patients from the different centres, between patients from different ethnic backgrounds, or between males and females. With respect to clozapine response, there were no significant differences in both the allele and genotype counts \( \chi^2 = 0.06, \; 1 \text{ df.} \; p=0.80 \) (two-tailed); \( \chi^2 = 1.21, \; 2 \text{ df.} \; p=0.55 \) (two-tailed), respectively] between responders and non-responders (See Table 1 this chapter). There was no deviation from Hardy-Weinberg equilibrium for the HTR6 polymorphism in both the responders and non-responders to clozapine. Our allele frequencies were also comparable to those published by Kohen et al. (1996).

Table 1: Allele and genotype counts and frequencies of the T→C 267 polymorphism in the 5-HT6 receptor gene in clozapine-treated patients

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T267</td>
<td>26 (14%)</td>
<td>25 (15%)</td>
<td>51 (15%)</td>
</tr>
<tr>
<td>C267</td>
<td>156 (86%)</td>
<td>139 (85%)</td>
<td>295 (85%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>182</td>
<td>164</td>
<td>346</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T 267</td>
<td>4 (4%)</td>
<td>2 (2%)</td>
<td>6 (3%)</td>
</tr>
<tr>
<td>T/C 267</td>
<td>18 (20%)</td>
<td>21 (26%)</td>
<td>39 (23%)</td>
</tr>
<tr>
<td>C/C 267</td>
<td>69 (76%)</td>
<td>59 (72%)</td>
<td>128 (74%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>91</td>
<td>82</td>
<td>173</td>
</tr>
</tbody>
</table>

\(^{a}\chi^2=0.06, \; 1 \text{ df.} \; p=0.80 \) (two-tailed) for clinical response relative to allele counts [ODDS RATIO for T267 association to non-response=1.08, 95% CI 0.57-2.05]. \(^{b}\chi^2=1.21, \; 2 \text{ df.} \; p=0.55 \) (two-tailed) for clinical response relative to genotype counts.
4.5 Discussion

The pro16leu HTR1A polymorphism was identified by Inayama et al. (1995) in a sample of unrelated Japanese bipolar affective disorder patients and healthy controls using single-stranded conformational polymorphism (SSCP) analysis. We have employed an allele-specific oligonucleotide (ASO) hybridization technique to examine this polymorphism in our sample of clozapine-treated patients. This is a quicker and more specific way of genotyping this polymorphism in comparison to SSCP. In our sample, all individuals genotyped were homozygous for the pro16 polymorphism. Inayama et al. (1995) found frequencies for the rarer leu16 variant of 2% and 31% in bipolar patients and controls, respectively. However, their control population was not observed to be in Hardy-Weinberg equilibrium for this polymorphism.

SSCP analysis examines mobility shifts in single-stranded PCR-amplified DNA when electrophoresed on polyacrylamide gels (Orita et al., 1989). Observed differences in mobility shifts between PCR-amplified DNA from different individuals, under specific conditions, may indicate the presence of a nucleotide substitution. That is, the nucleotide substitution changes the conformation of the single-stranded DNA, under specific conditions, and this causes differential migration rates between the single-stranded DNA from the alleles of polymorphic individuals. SSCP is a good technique to screen for point mutations, however, it is not specific enough for genotyping a specific polymorphism in a large sample of individuals, i.e. other polymorphisms may cause similar mobility shifts and these may be indistinguishable from each other. It is possible that Inayama et al. (1995) incorrectly genotyped some individuals in their sample for that reason, and this may
account for their observed deviation from Hardy-Weinberg equilibrium in their control sample.

This pro16leu HTR1A polymorphism may only exist in Asian populations and thus we were unable to detect it in our heterogeneous North American sample consisting predominantly of Caucasians and African-Americans. This polymorphism may be involved in clozapine response in the Asian population but not in other ethnic groups, thus future studies of this HTR1A polymorphism and response to clozapine in Asian samples may be warranted. We are currently in the process of genotyping other polymorphisms at the HTR1A locus in our sample of clozapine-treated patients.

The pro279leu variant at the HTR7 locus was observed in only one individual in our entire patient sample. This person was a 23 year old Caucasian male responder to clozapine. There were no outstanding clinical characteristics that distinguished this individual from any of the other responders to clozapine. This polymorphism is located within the third intracellular cytoplasmic loop of the 5-HT7 receptor protein and the substitution of pro279 to leu279, based on secondary structure analysis, is postulated to change local protein structure and thus affect coupling to G-proteins (Pesonen et al., 1995). Therefore the polymorphism may result in altered receptor function and thus may be involved in the phenotype of clozapine response. However, confirmation of this putative functional effect remains to be established. The rare occurrence of the leu279 variant suggests that even if functionality of this polymorphism is confirmed, it would only be playing a role in a very small number of patients and is thus not likely to be contributing in a major way to clozapine response. Studies, to date, have only identified rare polymorphisms within HTR7 with low informativeness (Erdmann et al., 1996b; Gelernter
et al., 1995; Pesonen et al., 1995). Thus additional polymorphisms that are more informative are required to test the hypothesis that genetic variation in the 5-HT7 receptor may predict response to clozapine.

We have found no evidence of an association between a silent polymorphism in the 5-HT6 receptor gene and clinical response to clozapine in schizophrenia patients. It is possible that linkage disequilibrium that may exist between this marker and a putative response locus may be masked by the heterogeneity of our North American sample thereby explaining our negative result. However, we checked to make sure that our responder and non-responder groups were as similar as possible with respect to ethnic composition. To our knowledge, this is the first report examining genetic variation in HTR6 and response to clozapine. Power analysis for this HTR6 polymorphism revealed that our sample has the ability to detect an odds ratio as low as 2.33 for a T267 association with non-response, specifying a type I error rate ($\alpha$)=0.05, power (1-\(\beta\))=0.80, and the proportion of responders with the hypothesized T267 risk allele for non-response ($P_0$)=0.11. Therefore, even if the T→C 267 HTR6 polymorphism was only contributing in a minor way to the overall variance observed in response to clozapine, our sample has the required power to detect an association.

Clinical response to clozapine is likely to be a complex genetic trait involving a variety of different factors. Environmental influences in combination with genetic variation in key proteins thought to interact with clozapine may be involved. This study has focused on genetic variability in receptors from the serotonin system whose products have affinity for clozapine, and are expressed in regions of the CNS implicated in the pathophysiology of schizophrenia. These molecular genetic association approaches allow
for the analysis of several potential candidate genes that may only contribute to a small amount of the total variance observed in the trait of clozapine response, and this approach may be useful in genetically dissecting this complex trait (Risch and Merikangas, 1996).

4.6 Statement of significance

This work has suggested that genetic variation in the 5-HT6 receptor may not be involved in the prediction of clozapine response. Identification of other polymorphisms at HTR1A, and HTR7 is recommended and these should be tested with respect to prediction of response to clozapine.
5.0 Summary and General Discussion
5.0 Summary and General Discussion

Response to clozapine, which is a highly variable phenomenon among psychiatric patients receiving this atypical antipsychotic, is likely to be a complex trait with many factors involved. From a clinical pharmacological perspective, both pharmacokinetic and pharmacodynamic considerations must be accommodated.

In considering clozapine’s pharmacokinetic profile, it is clear that there is a great deal of variability between individuals as steady-state plasma concentrations of clozapine vary widely between patients treated with the same oral dose (Dahl et al., 1994; Choc et al., 1990; Thorup and Fog, 1977; Bondesson and Lindstrom, 1988; Cheng et al., 1988). This would imply that inter-individual differences in the absorption, metabolism, elimination as well as transport across the blood-brain barrier may partially account for clozapine’s highly variable response profile. It is possible that genetic variation in biological structures involved in regulating these processes may be involved.

Traditionally, the study of pharmacogenetics has focused on polymorphisms in genes that encode enzymes which metabolize the drug with the variable pattern of response. With respect to clozapine, there has been no clear log-linear plasma level-response relationship demonstrated (reviewed in VanderZwagg et al., 1996), and there is a threshold (range: 350-400 ng/mL) for plasma clozapine levels to achieve good response (reviewed by Lindemayer and Apergi, 1996). The latter, in addition to the fact that there have been no polymorphisms found in CYP1A2 (Nakajima et al., 1994), the major metabolizer of clozapine, suggests that genetic-pharmacokinetic factors may be playing only a small role in the observed variability in response to clozapine.
More recently, several groups have suggested that inter-individual differences in the pharmacodynamics of a drug may be involved in producing the observed variability in its response profile (Kennedy, 1994; Kerwin et al., 1994; Propping and Nöthen, 1995). In considering clozapine, genetic variability in receptor proteins, for which it has high affinity, may account for some of the variation observed in its pattern of response. This approach has been the central focus of this thesis which has examined polymorphisms in genes from the serotonin system.

The hypothesis that genetic variation in serotonin receptors may predict response to clozapine has been tested in a large (n=185), clinically well-characterized sample of schizophrenia patients who were prospectively assessed for response to this atypical antipsychotic. We found evidence of both an allelic and genotypic association of the his452tyr polymorphism in the 5-HT2A receptor gene with clinical response to clozapine. Two other polymorphisms in this receptor gene, which were in strong linkage disequilibrium with each other, were not found to be associated with response. Combining all of the genetic information studied at the 5-HT2A receptor gene using a haplotype approach did not reveal any additional associations with response.

Genetic variation in both the 5-HT2C and 5-HT6 receptors did not predict response to clozapine. A pro16leu substitution in the 5-HT1A receptor, which was discovered in a Japanese sample, was genotyped in our sample and was not observed to be polymorphic. A pro279leu polymorphism in the 5-HT7 receptor was also assessed in our clozapine-treated patients and this was found to be uninformative; only one responder possessed the rare leu279 variant. Therefore, we were unable to draw any conclusions regarding the involvement of the 5-HT1A and 5-HT7 receptor genes in clinical prediction.
of clozapine response. Inter-ethnic differences in the genotype frequencies of the cys23ser polymorphism in the 5-HT2C receptor were also observed. African-Americans possessed a higher occurrence of the rarer ser23 variant than Caucasians.

Overall, our study supports the hypothesis that genetic variability in the 5-HT2A receptor gene is involved in the highly variable response profile of clozapine among individuals. The polymorphism that was found to be associated with the trait of response/non-response produced a difference in the amino acid sequence of the 5-HT2A receptor protein. A tyrosine residue was substituted for a histidine residue at position 452 in the carboxyl terminal tail of this receptor. We demonstrated that non-responders to clozapine were more likely to possess the rarer tyr452 variant than responders. Ozaki et al. (1997) have recently reported that the tyr452 HTR2A variant exists in a partially desensitized state in comparison to the his452 version. It is plausible that this functional difference between the HTR2A variants may account for our positive association with response to clozapine.

It is important to note here that the Phi coefficient (φ) was equal to 0.13. This coefficient provides an estimate of the strength of the association between the tyr452 variant and non-response to clozapine. φ ranges from 0 to 1 where a value of 1 indicates complete dependence between the rows (his452/tyr452) and columns (response/non-response) of the chi-square contingency table (Table 2b in Chapter 3); a value of 0 indicates complete independence. With respect to our demonstrated association, a φ of 0.13 suggests that there is only 13% agreement between the phenotype of non-response/response and the presence/absence of the tyr452 variant, respectively. In other words, the association is modest and 87% of the observed data cannot be accounted for
by the relationship between the tyr452 variant and non-response to clozapine. This would suggest that there are other factors playing a role in the phenotype of clozapine response/non-response, possibly polymorphisms in other genes. An attempt will now be made to establish a link between our observed association and the hypotheses put forth by Kapur and Remington (1996) described in Chapter 1. It is important to keep in mind that this will be highly speculative in nature and that no firm conclusions can be reached as a result of the association being only modest.

Based on the model presented in the general introduction (Figure 1), blockade of 5-HT2A receptors located on nigrostriatal dopaminergic neurons disinhibits the dopamine system leading to increased levels of dopamine at the striatum, and this in turn is thought to reduce EPS. A similar mechanism may be working on dopaminergic mesocortical projections to the prefrontal cortex, thus 5-HT2A blockade may also help to alleviate negative symptoms. Our study has assessed overall response to clozapine, and has not specifically considered EPS and negative symptoms. However, the Brief Psychiatric Rating Scale (BPRS), employed to assess response to clozapine in this thesis, does include measures of negative symptoms in its ratings. In other words, some patients considered to be responding to clozapine (i.e. 20% decrease in total BPRS) may be doing so as a result of improvement in their negative symptomatology which would be reflected, in part, by the total BPRS score.

In order to relate our finding of a positive association between the his452tyr HTR2A polymorphism and clozapine response to the serotonin-dopamine interaction hypothesis of atypical antipsychotic action proposed by Kapur and Remington (1996), several assumptions must be made. The assumptions are as follows: 1.) responders to
clozapine were less likely to exhibit EPS than the non-responders, 2.) responders were more likely to be improved with respect to their negative symptoms than non-responders, 3.) our result was a true positive, and 4.) the findings of Ozaki et al. (1997) were true and occur in vivo.

We observed that non-responders to clozapine were more likely to possess the rarer tyr452 variant than responders. Ozaki et al. (1997) reported that the tyr452 receptor variant exists in a partially desensitized state compared to the his452 variant. Using the model presented by Kapur and Remington (1996) schematically depicted in Figure 1, and considering only homozygous individuals, tyr/tyr 452 HTR2A individuals should have a somewhat disinhibited nigrostriatal dopaminergic system in comparison to his/his 452 individuals; dopamine levels should be slightly higher in these tyr/tyr 452 individuals. That is, desensitized 5-HT2A tyr452 receptors are not as responsive to serotonin as 5-HT2A his452 receptors and, as a result, the extent of serotonergic inhibition of the dopamine system is less in tyr/tyr 452 individuals. Hypothesis 1 in Figure 2 predicts that, with the addition of clozapine or other atypical antipsychotic possessing a high ratio of 5-HT2A:D2 blockade, EPS is reduced because of the increased competition of dopamine with the antipsychotic at D2 receptors in the striatum.

Our results are inconsistent with hypothesis I because individuals possessing the tyr452 variant should have an advantage over his/his 452 individuals with respect to reduced EPS, even without 5-HT2A blockade, as a result of constitutively reduced 5-HT2A activity. Therefore, tyr/tyr 452 individuals should more likely be responders to clozapine compared to his/his 452 individuals. With regards to negative symptoms, the
same approach can be used to argue that tyr/tyr 452 individuals should more likely be responders to clozapine. This is contrary to what we observed.

Although it is difficult to speculate as to what exactly is going on, hypothesis II in Figure 2 (Chapter 1) may perhaps provide answers. Hypothesis II proposes that 5-HT2A blockade by atypical antipsychotics elevates the EPS threshold through modulation of GABAergic or cholinergic mechanisms without having a direct effect on D2 occupancy. Further work is required to elucidate possible mechanisms of this interaction and thus we are unable to put our results into the perspective of hypothesis II (Figure 2).

Pharmacogenetic prediction of response to clozapine from a pharmacodynamic perspective is a relatively novel approach to examine its variable response profile. To our knowledge, there are currently only four groups in the world, including ours, attempting to identify polymorphisms involved in this trait. For the most part, the results from the different groups have been inconsistent. The reason for this may be attributed to differences in both the clinical and genetic methodology employed by the different groups.

With respect to differences in clinical methodology, previous studies of the pharmacogenetics of clozapine response were conducted retrospectively; response was ascertained through the review of medical records. These studies also did not monitor blood levels of clozapine, such that the issues of compliance or under-treatment are brought into question. This factor is extremely important considering that there is a threshold plasma clozapine level which predicts good response (reviewed by Lindenmayer and Apergi, 1996). Some of the previous studies did not maintain clozapine treatment for a sufficient period of time which is quite important given the observation that some patients’ response to clozapine may be delayed until six months (Meltzer, 1989b, 1995b;
Lieberman et al., 1994). Our studies have attempted to circumvent these problems by including patients that were assessed prospectively for response to clozapine over a period of six months. Our patients were also evaluated in psychopharmacology research clinics that have extensive experience in measuring response to antipsychotic medications. The definition of response employed in our sample was perhaps the 'gold standard' of the time during which the clinical trials took place (Kane et al., 1988). Furthermore, clozapine blood levels of the patients were carefully monitored at regular intervals to ensure that all of the patients achieved the required plasma level threshold for response.

With respect to differences in the genetic methodology among the groups, all of the previous studies have examined the contribution of only one candidate gene polymorphism at a time. By employing a haplotype approach, we combined all the available genetic information at the HTR2A locus and examined the possibility of particular haplotypes being associated with response. In this way, with regards to the phenotype of clozapine response, we have increased our probability of detecting the contribution of polymorphisms that have not yet been identified at HTR2A as this approach covers a large region of the gene. We also have demonstrated that our sample has adequate power to detect genes of small effect. As a result of small sample sizes, some of the previous studies did not possess adequate power to detect a significant gene effect even if there was one.

There were several limitations with our study. First of all, and probably most important, was the ethnic composition of our sample. Our patient samples were collected at three clinical sites in the U.S., and as a result there was a great deal of ethnic diversity. Therefore, our sample was ethnically heterogeneous. Although we tried to control for this
confounding factor by ensuring that our responder and non-responder groups were ethnically similar, this does not exclude the possibility that our positive result was due to population stratification. Another limitation of our study is multiple testing. Replication in independent samples is required to confirm our finding. With respect to our his452tyr finding, the fact that Arranz et al. (1996) have observed a similar result in their sample provides additional support for our finding.

The way that response to clozapine was defined in our sample is also a limitation. When patients are categorized as responders and non-responders to a medication based on a cut-off score, in our case a 20% decrease in BPRS, individuals falling in the range of 15 to 20% may demonstrate marked improvement to clozapine overall, despite not actually achieving the cut-off. We attempted to circumvent this problem by using the Clinical Global Impressions (CGI) scale to augment the definition of response in these questionable cases. Despite this, important clinical information is lost when categorizing individuals into groups.

The overall significance of this work will now be discussed. Based on our group's studies for the past four years and my studies for the past three, several recommendations for future pharmacogenetic studies of clozapine response will be made. These have been based on a critical review of the previous studies, and a critical examination of our own work. The recommendations are as follows:

1.) All groups working in this field should agree upon a standard definition of response a priori, and this should include multiple clinical criteria. Furthermore, rather than using a categorical approach with non-parametric statistics, it would be advisable to use the
actual fractional change in clinical score over time in an analysis of variance (ANOVA) with genotype as the grouping factor.

2.) The duration of the clinical trial should be long enough to ensure that the patients have an adequate response rate (between 40 and 60%). In the case of clozapine, this should be in the range of 5 to 6 months.

3.) The clinical studies should be conducted prospectively, and sample size calculations should be done *a priori*, taking into consideration allele frequencies for the specific polymorphism that is being examined. Plasma levels of clozapine should be assessed to ensure that patients are compliant and that they achieve the plasma cut-off which predicts good response. This should reduce the confounding effects of inter-individual differences in pharmacokinetic factors.

4.) Clozapine response should be assessed in genetically homogeneous and isolated populations to reduce the confounding factor of population stratification. Other demographic factors including sex, and age should be taken into consideration, e.g. select patients within a certain age range.

5.) Multiple polymorphisms across a given locus should be examined so that a haplotype approach can be used. This ensures that the entire gene in question is studied and thus the effects of other polymorphisms not yet identified may be determined.

6.) Any positive results should be replicated in independent samples using exactly the same criteria as the original study.
6.0 Conclusions
6.0 Conclusions

This study has provided evidence supporting the hypothesis that genetic variation in receptors for which clozapine has affinity predicts response to this atypical antipsychotic. A polymorphism that leads to a his452tyr amino acid substitution in the 5-HT2A receptor was demonstrated to be associated with response to clozapine. Future in vitro work with this particular polymorphism should include expression of the 5-HT2A receptor variants in cell lines that have been transfected with either version of this receptor, followed by subsequent pharmacological and functional characterization. The advantage of this method in comparison to that of Ozaki et al. (1997) is that natural sources of inter-individual variation in the Ozaki platelet model, which may confound the results, can be controlled for in the transfection model. The functionality of the variants should be examined in both the presence and absence of clozapine to determine its specific actions in each of the transfected systems. Future in vivo work with this particular polymorphism should include positron emission tomography (P.E.T.) studies in individuals homozygous for the his452 or tyr452 variants.

Regarding genetic prediction of response to clozapine, it would be useful to screen for novel mutations in other candidate genes using SSCP and genotype these in our sample. This is warranted as our study supports the concept that response to clozapine is complex and multi-factorial. Our studies have focused on pharmacodynamic-genetic factors thought to be involved in clozapine response. Pharmacokinetic-genetic factors may also play a role in clozapine's variable response profile and thus screening for mutations in CYP1A2, the major metabolizer of clozapine, as well as regulators of
CYP1A2 expression, such as the Aromatic Hydrocarbon (Ah) receptor may identify common polymorphisms that can be genotyped in our sample.

Other interesting future work should examine clozapine's effects on specific symptom clusters. These studies should aid in the genetic dissection of the complex trait of clozapine response. Examination of genetic prediction of side effects to clozapine should also provide useful information. The side effect of clozapine-induced weight gain is particularly interesting as a candidate from the serotonin system, the 5-HT2C receptor, may be involved. Tecott et al. (1995) have observed that mice lacking the 5-HT2C receptor are overweight compared to wild-type mice and this is thought to be due to increases in feeding behaviour. Examination of the cys23ser HTR2C mutation may provide some useful insight into the side effect of clozapine-induced weight gain.

In order to further address the issue of population stratification in genetic studies, it may be useful to employ a Haplotype Relative Risk (HRR) approach to examine the phenotypes of response or non-response. This approach takes individuals who are either responders or non-responders to clozapine as well as their parents. Hence one sample would consist of clozapine responders and both of their parents while the other would consist of clozapine non-responders and both of their parents. Polymorphisms in candidate genes are then examined in these response and non-response HRR triads (proband and both of their parents) and the distribution of transmitted and non-transmitted alleles are compared in each group. Essentially, the untransmitted chromosomes from each of the parents 'makes up' the control genotype. This method should effectively eliminate any problems of population stratification as the proband is identical ethnically to his/her parents (Thomson, 1995).
From these pharmacogenetic studies one or more genetic factors may be discovered that play an important role in antipsychotic medication response. This genetic information may aid clinicians in deciding who should receive a particular drug, and it may also help in elucidating the mechanism of action of these atypical antipsychotic agents. Ultimately, this information may lead to the design of new, more specific therapeutic agents, and may illuminate our understanding of the pathophysiology of schizophrenia.
7.0 References
7.0 References


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8.0 Appendix: Other publications
8.1 Absence of Linkage for Schizophrenia on the Short Arm of Chromosome 5 in Multiplex Canadian Families

Nicole King, Anne S. Bassett, William G. Honer, Mario Masellis, James L. Kennedy
American Journal of Medical Genetics (Neuropsychiatric Genetics) (1997); 74: 472-474

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KEY WORDS: schizophrenia, schizotypal personality disorder, linkage, dopamine transporter, chromosome 5p
ABSTRACT

A VNTR for the human dopamine transporter gene (DAT-1) has been localized to chromosome 5p15.3. Silverman et al., [1996] found evidence for genetic linkage of the D5S111 locus, located just centromic to DAT-1, to schizophrenia and related disorders in a large Hispanic family. We evaluated five markers on 5p, including D5S111 and the DAT-1 VNTR in five multiplex schizophrenic families, assuming autosomal dominant transmission (subjects assessed n=122, DNAs available n=96, individuals with schizophrenia and schizoaffective disorder n=36, broader spectrum disorders n=14). Lodscores were negative across all families for all markers tested, and overall lodscores were strongly negative (< -2.0, =0) across all five families for each of the markers typed. Thus, there is no evidence to support the linkage of markers in this region of chromosome 5 to schizophrenia in this sample of families.

INTRODUCTION

The cause of schizophrenia is as yet unknown, however family, twin and adoption studies carried out over the past half century consistently indicate that genetic factors are involved in the etiology of this disease [Gottesman and Shields, 1982]. There is a well-documented role for dopamine in schizophrenia [Losonczy et al., 1987]. One model which could contribute to functional dopamine abnormalities is the dopamine transporter, which is responsible for termination of the activity of synaptic dopamine through re-uptake [Hitri et al., 1994]. A genetic association study indicated a potential role for the dopamine transporter gene (DAT-1) in the etiology of psychosis in cocaine abusers [Gelernter et al.,
Silverman et al. [1996] have recently reported evidence for genetic linkage of a locus near DAT-1 (D5S111, 5p14.1-13.1) to schizophrenia in one large pedigree. A maximum lod score of 3.72 (q = 0.01) was found assuming autosomal dominant inheritance. Based on the above rationale, we were prompted to examine markers on the short arm of chromosome 5, including a VNTR for the dopamine transporter gene (DAT-1).

**MATERIALS AND METHODS**

Blood samples from five eastern Canadian families were collected by one of us (ASB). Diagnosis was established (including: schizophrenia, schizoaffective disorder, psychosis NOS, schizotypal personality disorder, or paranoid personality disorder) using the Structured Clinical Interview for DSM-III-R (SCID-I, SCID-II), and available medical records [Bassett et al., 1993, Bassett and Honer, 1994]. Following from Silverman et al. [1996], we examined the broad spectrum of schizophrenia as the principal phenotype. Genomic DNA was extracted using the high salt method. We genotyped four highly informative microsatellite markers (D5S117, D5S108, D5S111, D5S411) and the dopamine transporter gene (DAT-1) VNTR, all of which are distributed across the short arm of chromosome 5. The DAT-1 VNTR was typed according to the method of Vandenbergh et al. [1992]. For the microsatellite markers, the left primer was end-labelled with gamma-32P ATP and T4 polynucleotide kinase. PCR products were separated by electrophoresis on 6% polyacrylamide gels. Autoradiography was performed overnight and genotypings were analyzed by laboratory staff blind to diagnoses. Allele frequencies were derived from unrelated individuals among the families. LOD scores for
pairwise analyses were generated using the MLINK program of the LINKAGE package, version 5.2. LOD scores were evaluated under the assumption of an autosomal dominant mode of inheritance (gene frequency = 0.85%, phenocopies = 0.001, penetrance = 70%).

RESULTS

The results of our lod score analyses are shown in Fig. 1. We were unable to replicate the positive lodscore of Silverman's group or to generate any suggestive positive lodscores in any of the five families analyzed for D5S111 or any of the other flanking markers. Overall lodscores were strongly negative across all five families for each of the microsatellite markers genotyped, resulting in an exclusion region (lod score < -2.0) extending at least 10 centimorgans on either side of each marker. For the candidate gene DAT-1, linkage was excluded out to a 2 cM region flanking the marker. A recessive model was tested and also yielded strongly negative results for all markers.

DISCUSSION

We obtained negative results for each of the five markers that we tested on chromosome 5p. The discrepancy between our findings and those of Silverman et al. may be due to a chance result in their study since they were not able to replicate this positive lodscore in any of their other 22 families, or our result could be a false negative. Another possible explanation is the problem of locus heterogeneity which is probably present in most complex genetic disorders. It appears highly likely that there is more than one susceptibility locus for schizophrenia in the human genome. The family studied by Silverman et al. is unique in that it has its origins in a relatively isolated rural area of
Puerto Rico and thus the genetic variant causing schizophrenia within this population would be expected to demonstrate a higher degree of genetic homogeneity [Vazquez Calzada, 1981]. The limitations of our study are as follows: only five families were analyzed, and we did not study an Hispanic racial subgroup therefore our study cannot be considered a closely-related replication attempt. Alternatively, the original findings may have been dependent upon the use of different diagnostic methods. Given the numerous arenas in which genetic studies can differ, and given continued interest in DAT-1 as a candidate gene, other investigations of 5p, particularly in homogeneous populations, may be of interest.

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Fig. 1. Pairwise analysis between schizophrenia and chromosome 5 markers. Map distances were obtained from the database at the website of the University of Southampton Genome Center (www.cedar.genetics.soton.ac.uk).

**Pairwise Analyses of 5p Markers and Schizophrenia**