THE GLUTAMATE RECEPTOR GENES AND SCHIZOPHRENIA: GENE EXPRESSION STUDIES OF ANTIPSYCHOTIC DRUG ACTION AND LINKAGE ANALYSIS OF THE GENETIC SUSCEPTIBILITY

by

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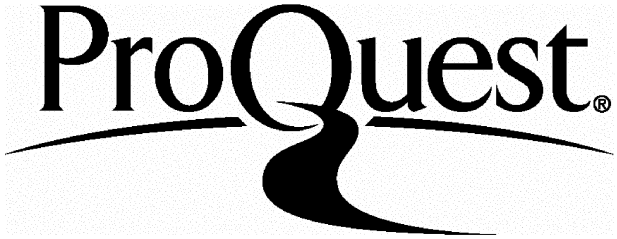

DEDICATION

To my parents Mr. and Mrs. Yao-Nan and Yueh Chen

and

sister Alice Ching-Hui Chen

ABSTRACT

Schizophrenia is a severe disabling neuropsychiatric syndrome affecting 0.85% of most human populations. Family, twin, and adoption studies in several ethnic populations have consistently implicated genetic factors as having an important pathogenic role in the disease. However, researchers have only recently begun to detect consistent evidence of linkage between schizophrenia and genetic markers, but no candidate genes have yet been implicated.

It has been hypothesized that glutamate receptor function is important in either the aetiology or treatment of schizophrenia and therefore the glutamate receptor family of genes are potential susceptibility loci for schizophrenia. To test this hypothesis, twenty-three English and Icelandic schizophrenia families containing multiple cases of schizophrenia were genotyped with currently available microsatellite polymorphisms localized at the GluR5, GluR6 glutamate receptors and SLC1A5 glutamate/aspartate transporter loci. Lod scores, model-free linkage analysis, and extended relative pair analysis methods were used to test for linkage. No evidence of close linkage between schizophrenia and any of these loci was found.

In addition, in order to understand how specific glutamate receptor genes are involved in the treatment of schizophrenia, a multiprobe oligonucleotide solution hybridization (MOSH) technique was used to examine the regulation of gene expression of the ionotropic glutamate receptor subunit genes. Four regions of the left rat brain following treatment with the optical isomers of flupenthixol at a dose of 0.2 mg/kg/day over a period of 1, 2, 4, 8, 12, 24 weeks were studied. A previous controlled trial showed that cis-flupenthixol had a significantly superior ability to ameliorate the positive symptoms of schizophrenia compared to its trans-isomer. Protein levels of the NMDA receptor subunit NR1, GluR2/3 and GluR6/7 glutamate receptors in the right brain were also examined by Western blotting technique with specific antibodies for these receptor subunits.

In summary, the gene expression of specific NMDA receptor subunits in several regions of the left rat brain was altered by treatment with either the cis- or trans-isomer of the antipsychotic drug flupenthixol. NR1 mRNA was significantly decreased throughout the 24 weeks treatment with trans- flupenthixol and after long-

term (12 or 24 week) treatment with cis- flupenthixol in the frontal and subcortical areas. NR2B and NR2C mRNA expression demonstrated a dynamic pattern of change in different brain regions following treatment with flupenthixol whilst NR2A and NR2D gene expression was relatively unaffected except in the subcortical region. The gene expression of AMPA (GluR1-4) and kainate (GluR5-7, KA1, KA2) types of glutamate receptor subunits was unaffected following 4 and 24 weeks of treatment with either trans- or cis- flupenthixol. It would be difficult to make inferences about the pathophysiology of schizophrenia or any other psychiatric disorders solely based on drug mechanisms. However, these results indicate that adaptations in glutamate receptors may represent an important and novel mechanism through which neuroleptics exert some of their effects on brain function.

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ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

ATP: adenosine triphosphate

bp: base pair

cDNA: complementary DNA

CEPH: Centre d'Etude du Polymorphisme Humain

cM: centiMorgan

CPP: (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid

dATP: deoxyribosyladenine 5' triphosphate

dCTP: deoxyribosylcytosine 5' triphosphate

df: degree of freedom

dGTP: deoxyribosylguanine 5' triphosphate

dTTP: deoxyribosylthymine 5' triphosphate

DNA: deoxyribonucleic acid

DSM IV: diagnostic statistical manual, the 4th ed.

DTT: dithiothreitol

EDTA: ethylenediamine tetra acetic acid

GDB: genome database

HLA: human leukocyte antigen

hrs: hours

IBD: identical by descent

KA: kainic acid (kainate)

kb: kilobase pair

lod: logarithm of odds

min: minute

ml: millilitres

MOSH: multiprobe oligonucleotide solution hybridization

mRNA: messenger RNA

ug: micrograms

ul: microlitres

ng: nanogram

NMDA: N-methyl-D-aspartate

PBS: phosphate buffered saline

PCP: phencyclidine

PCR: polymerase chain reaction

PIC: polymorphism information content

RDC: research diagnostic criteria

RFLP: restriction fragment length polymorphism

RNA: ribonucleic acid

RNase: ribonuclease

SADS-L: schizophrenia and affective disorders schedule (lifetime version)

SDS: sodium dodecyl sulphate

SSC: standard sodium citrate

Tris: tris-(hydroxymethyl)aminomethane

V: volts

VNTRs: variable number of tandem repeats

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CHAPTER 1: OVERVIEW OF SCHIZOPHRENIA

Schizophrenia is a severe disabling neuropsychiatric syndrome with a life time expectancy of 0.85% in most human populations (Eaton 1985). There is no obvious difference in the prevalence between men and women. However, there are gender differences in age of onset, that is, men tend to develop this disorder at an earlier age (mean = 26.2, sd = 7.1) than women (mean = 31.3, sd = 9.9). It is most prevalent in lower social classes and inner city areas and 10 percent of schizophrenic patients commit suicide (Faraone et al., 1994; Tsuang et al., 1991). However, schizophrenia should by no means be considered a disorder of modern society. The Hindu *Ayurveda* (1400 BC) contains brief descriptions of an illness resembling schizophrenia, and may represent the earliest recording of the major psychoses in society (Kendell 1993). At the turn of this century the major advances in clinical psychiatry resulted from work by a few German physicians who were convinced that mental illnesses were disorders of the brain. They were the first to attempt to classify the psychoses and initiated modern phenomenology of mental illness and recognised that the natural history of mental illness had to be adequately delineated. For example they recognised catatonia as a type of psychosis with a motor disorder consisting of odd movements and stupor associated with mental deterioration. Subsequently, they introduced the terms cyclothymia for circular insanity and hebephrenia for a rapid mental deterioration occurring during puberty.

Emil Kraeplin (1855-1926) was probably one of the most outstanding psychiatrists among these German pioneers. He developed a classification for the mental disorders using clinical observation as well as an awareness of natural history. He described an illness with typical symptoms of hallucinations, delusions and thought disorder and then grouped these disorders as subtypes of a single disease entity ubiquitously called "dementia praecox" in his textbook of Psychiatry in 1899. He categorized the illness into hebephrenic, catatonic and paranoid types according to symptoms and the medical history of the illness. In 1913, he added another type of "dementia simplex" to the classification system. In 1893 he recognised a separate psychosis which had a periodic nature consisting of manic illness and depressions. He grouped recurrent mania, depressions and circular insanity as manic-depressive

insanity. This was the first recorded separation of the two major psychoses namely schizophrenia and affective disorders which has been of primary importance for the development of modern classification systems used in clinical psychiatry.

The Swiss psychiatrist, Eugen Bleuler (1857-1939) in 1911 introduced the term "schizophrenia" which means "splitting of the mind" for the disease because he believed that the functions of the mind were split off from each other in the disease. The "split" in schizophrenia was referred to abnormal association between thoughts, emotion and behaviour, but not "split personality". He described four basic symptoms of schizophrenia: abnormal association between thoughts, abnormality of affect, morbid ambivalence and autism. He was also the first clinical psychiatrist to apply Freud's theories of psychotherapy to the study of psychotic symptoms.

Another influential German psychiatrist, Kurt Schneider, described the first-rank symptoms of schizophrenia as those disturbances of experience including audible thoughts, voices arguing and/or discussing, voices commenting, somatic passivity experiences, thought withdrawal and broadcasting, delusional perceptions and feelings, impulses and volitional actions imposed by an external force. Schneider's diagnostic system played an important role in many subsequent elaborations of diagnostic criteria, such as the International Classification of Diseases (ICD), the Research Diagnostic Criteria (RDC), and the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria which are currently the most commonly used criteria for diagnosing mental illness.

Modern clinical psychiatry has concerned itself with improving diagnostic systems, psychopharmacology, and research to elucidate the true aetiological factors for the major psychosis. It is crucial to understand that in the absence of any biological diagnostic marker, concepts of schizophrenia might eventually prove to be misleading. Nevertheless comparison of the current concepts with descriptions of madness in the early nineteenth century indicates that the major clinical features have remained constant. However, because of the rapid development of antipsychotic drugs in the past forty years and modern medical practices, the symptoms of schizophrenia seem to have become less severe and bizarre in recent years with some investigators claiming that the first admission rate for the disease is diminishing (Murray, 1990).

1.1 Clinical features and symptoms of schizophrenia

Schizophrenia has an onset in early adult life. The main symptoms of schizophrenia consist of a disorder of thought, perception, emotion, and abnormal ideas. These occur in symptom complexes, but for the convenience of lucidity they will be considered individually even though this is somewhat artificial.

1.1.1 Disorders of thought content and thought process

Abnormalities in thought can be divided into those of content such as ideas, beliefs, and interpretations of stimuli, as well as those of process and form observed in the spoken language and in how ideas and language are formulated.

Delusions are false beliefs and are examples of a disorder of form and content. Often bizarre they may be persecutory, grandiose, religious or somatic. Schizophrenics can sometimes believe that some outside force is controlling their thoughts and behaviours or that they are controlling outside events in some extraordinary fashion. Paranoid delusions commonly observed in patients are that they are being spied upon, talked about or at risk from being harmed. Patients may also experience thought broadcasting, thought insertion, thought withdrawal, and thought control. The common central theme of schizophrenic delusions is the direct, immediate and total certainty with which a patient holds these beliefs.

Schizophrenic speech has a tendency to be filled with bizarre or symbolic images whose meaning is difficult to be comprehended. Speech is sometimes preoccupied with abstract, psychological and philosophical ideas. Patients may lack a clear sense of where their own body, mind and influence ends and where these characteristics in other animate and inanimate objects begin. For example, the patient may believe that other people or the television are talking about them or that they have fused with another object or disintegrated completely.

Abnormalities of the form of thought are observable in the spoken and written language of the patient whilst those of the process of thought refer to how ideas and language are formulated in the brain. Consequently, the two are interrelated. Forms of thought abnormalities found in schizophrenia include loosening of

associations, incoherence, tangentiality, flight of ideas, neologisms, echolalia, verbigeration, mutism, thought blocking, impaired attention, over inclusion, illogical ideas, vagueness and poverty of speech content. The patients however do not seem to be aware that their communication is abnormal.

1.1.2 Perceptual disturbances

Hallucinations and delusions are very common in schizophrenia. On the other hand illusions are common but also experienced by many normal people though to a lesser extent. An illusion is a misinterpretation of a sensory stimulus, while a hallucination is a perception in the absence of an actual external stimulus.

Hallucinations can occur in any of the five sensory modalities, however up to 75% are auditory. Most auditory hallucinations in schizophrenics are voices of "the third person", that is, they may consist of voices of God or the devil; sometimes they are voices of neighbours, deceased relatives or unrecognised individuals. The schizophrenic may experience two voices discussing himself in the third person; voices may make obscene comments about the patient, and the patient may hold audible conversations with the voices. Hallucinations of smell are not uncommon; the patient complains of gas, odours of decomposition, chemical smells and so on and these are normally intertwined with their paranoid delusions. A third frequently found hallucination is of the bodily or somatic kind. Patients experience induced sensations of heat, cold, pain, or electric shock. In the acute or chronic schizophrenics hallucinations can be bizarre with feelings that their flesh is being torn away, their bowels are torn out, animals or machines are inserted into their bodies. Visual and gustatory hallucinations are relatively rare in schizophrenia and can be confused with strongly held delusions.

Illusions are difficult to differentiate clinically from hallucinations but perceptual hypersensitivity to light, sound, touch, smell, taste can occur. For example small changes in the lighting on another person's face might be perceived as a dramatic change by a patient and interpreted in a delusional way.

1.1.3 Inappropriate mood, feelings and affect

Mood and feelings of schizophrenics can be grossly reduced, extremely

exaggerated, or patently bizarre. Many patients show reduced emotional responses and seem to be indifferent with emotional shallowness. An extreme form of this could result in a profound emotional barrenness in which the patient is incapable of experiencing any pleasure. The emotional responses of schizophrenics are very often inappropriate to the situation. For example, they may smile whilst talking about a morbid subject or show unaccountable anger. Schizophrenics also suffer bizarre emotions with states of exaltation, feelings of omnipotence, and religious ecstasy. The patients may also show marked sensitivity to emotional trauma, being easily hurt by very mildly aggressive or rejecting behaviour by others.

1.1.4 Abnormal behaviours

First impressions of a schizophrenic can sometimes consist of extreme bizarreness, agitation or withdrawal, exhibited as a set facial expression, lack of sustained eye contact and staring at inanimate objects. Their personal appearance tends to deteriorate and they may exhibit idiosyncratic manners or offensive behaviour. Lack of motivation and will is demonstrated by an inability to continue an occupation or showing complete disinterest in future plans. In chronic patients stereotypic behaviour may sometimes present as repetitive patterns of moving or walking, strange gestures, or endless repetitions of the same phrase or question. Social withdrawal is a very common symptom in schizophrenia; contacts feel unable to establish rapport with the patient which often prevents others from feeling empathy or sympathy towards them. Until the mid-1950s when the antipsychotic drug chlorpromazine was developed, mental hospitals contained many severely affected patients with catatonic symptoms, stereotyped behaviours and grossly disorganized behaviour. Although catatonia, which is thought to be the severest form of schizophrenia, still occurs today, chlorpromazine and other new antipsychotic drugs have dramatically reduced many disabling symptoms of schizophrenia.

1.1.5 Course of the illness

Onset of schizophrenia before the age of 10 is rarely reported, whilst onset after 45 is found more often in women. Early features of schizophrenia may involve

patients feeling overwhelmed by external and internal pressures resulting in anxiety, irritability, distractibility, and impaired performance at work. Onset may also be characterised by boredom, apathy, hopelessness, loneliness, and unexplained aggressive behaviours. The stage of actual psychosis may occur after many quite obvious changes in general behaviour. Even then delusions can be hidden by denial and paranoid ideation.

The classical course is one of remissions with a lack of return to the patient's previous norm. Hallucinations, bizarre behaviour, sleeping problems, problems in thinking clearly and lack of self care are all common signs of a relapse. On average the deterioration in the quality of life continues for many years by which stage a plateau is reached. The positive symptoms tend to become less severe with time and the patient is left with the more socially and functionally debilitating negative symptoms. Many schizophrenics remain in a state of stable chronicity with clearly visible signs and symptoms of severe mental illness. 20-30% recover to lead relatively normal lives, a similar number continue to experience moderate symptoms whilst 40-60% remain significantly impaired for life (Sadock et al., 1989).

1.2 Classification and diagnostic criteria of schizophrenia

The past thirty years has witnessed a renaissance of clinical research on the diagnosis and classification of mental disorders. The classification of mental disorders into discrete categories has been a prerequisite for the scientific study of mental disorders. In addition, the introduction of antipsychotic drugs was also a spur to improve differential diagnosis between schizophrenia and mood disorders in the light of evaluation of their relative treatment responses. The emergence of laboratory and familial genetic strategies for exploring potential aetiological factors has benefitted from the use of consistent diagnostic criteria. Improved diagnosis has resulted from determining whether symptoms cluster into characteristic patterns or syndromes. As a consequence operationalized criteria have been developed in order to increase the reliability of diagnosis as well as to improve diagnostic validity and stability.

1.2.1 Diagnostic criteria

Modern diagnostic schemes were developed out of the earlier nosological theories of Kraepelin and Bleuler. The major diagnostic systems for schizophrenia include Schneider's (Schneider, 1959), the International Classification of Disease (ICD-10, WHO 1989), the St Louis Criteria (also called Feighner's criteria, Feighner, 1972), the Research Diagnostic Criteria (RDC, Spitzer et al., 1978a), the Present State Examination (PSE/CATEGO, Wing et al., 1974), and the Diagnostic Statistic Manual, 4th ed. (DSM-IV, American Psychiatric Association, 1994). All of these systems include the symptoms of psychosis in their criteria. The St Louis and DSM IV criteria require a reduced level of functioning for the diagnosis. The RDC, St Louis and DSM IV systems stipulate minimum duration of symptoms, and the St Louis system requires onset of symptoms before the age of 45. The DSM-IV criteria, which are the most commonly used, are listed in Table 1.1.

Table 1.1 Diagnostic criteria for schizophrenia (DSM-IV, 1994)

A. Characteristic symptoms: Two or more of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):

(1) delusions

(2) hallucinations

(3) disorganized speech (e.g. frequent derailment or incoherence)

(4) grossly disorganized or catatonic behaviour

(5) negative symptoms, i.e. affective flattening, alogia, or avolition

Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behaviours or thoughts, or two or more voices conversing with each other.

B. Social/occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations or self-care, are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve

expected level of interpersonal, academic, or occupational achievement).

C. Duration: Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e. active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g. odd beliefs, unusual perceptual experiences).

D. Schizoaffective and Mood Disorder exclusion: Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either (1) no major depressive, manic, or mixed episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g. a drug of abuse, a medication) or a general medical condition.

F. Relationship to a Pervasive Developmental Disorder: If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least 1 month (or less if successfully treated).

1.2.2 Subtypes of schizophrenia

All the diagnostic systems for schizophrenia are based on the presence of particular symptoms, course of illness, various exclusion criteria, and information drawn from the patient, family, past psychiatric status, and present mental status. The subtypes of schizophrenia are defined by the predominant symptomatology at

the time of evaluation. Although the prognostic and treatment implications of the subtypes are variable, the paranoid and disorganized types tend to be the least and most severe, respectively. The DSM-IV systems list five subtypes named paranoid type, disorganized type, catatonic type, undifferentiated type, and residual type.

Paranoid type

Characterized by delusions of persecution or grandeur, it has a relatively later age of onset and less deterioration of thought and social behaviour and emotional response.

The DSM-IV criteria for paranoid type are as follows:

A. Preoccupation with one or more delusions or frequent auditory hallucinations.

B. None of the following is prominent: disorganized speech, disorganized or catatonic behaviours, or flat or inappropriate affect.

Disorganized type

The essential features of the disorganized type of schizophrenia are disorganized speech, behaviour and flat or inappropriate affect. The disorganized speech may be accompanied by silliness and laughter that are not closely related to the content of the speech. The behavioural disorganization, i.e., lack of goal orientation, may lead to severe disruption in the ability to perform activities of daily life. This subtype is also usually associated with poor premorbid personality, early and insidious onset, and a continuous course without significant remissions. Historically, and in other classification systems, this type is termed "hebephrenic".

The DSM-IV criteria for disorganized type are as follows:

A. All of the following are prominent:

(1) disorganized speech

(2) disorganized behaviour

(3) flat or inappropriate affect

B. The criteria are not met for "Catatonic type" (see below).

Catatonic type

The essential feature of the catatonic type of schizophrenia is a marked psychomotor disturbance that may involve motoric immobility, excessive motor activity, extreme negativism, mutism, peculiarities of voluntary movement, echolalia, or echopraxia. In catatonia the patient may be in a state of complete stupor with waxy flexibility, stereotypes and a pronounced decrease in spontaneous movements and activity may also occur. The reverse is excited catatonia in which patients are in a state of extreme psychomotor agitation and talk and shout almost constantly.

The DSM-IV criteria for catatonic type are as follows:

A type of schizophrenia in which the clinical picture is dominated by at least two of the following:

(1) motoric immobility as evidenced by catalepsy (including waxy flexibility) or stupor

(2) excessive motor activity (that is apparently purposeless and not influenced by external stimuli)

(3) extreme negativism (an apparently motiveless resistance to all instructions or maintenance of a rigid posture against attempts to be moved) or mutism

(4) peculiarities of voluntary movement as evidenced by posturing (voluntary assumption of inappropriate or bizarre postures), stereotyped movements, prominent mannerisms, or prominent grimacing

(5) echolalia or echopraxia

Undifferentiated type

The essential feature and criteria of this type is the presence of symptoms that meet Criterion A (Table 1.1) of schizophrenia but that do not meet criteria for the paranoid, disorganized, or catatonic type.

Residual type

The residual type of schizophrenia should be used when there has been at least one episode of schizophrenia, but the current clinical picture is without prominent

positive psychotic symptoms, e.g., delusions, hallucinations, disorganized speech or behaviours. There is continuing evidence of the disturbance as indicated by the presence of negative symptoms, e.g., flat affect, poverty of speech, or avolition or two or more attenuated positive symptoms, e.g., eccentric behaviour, mildly disorganized speech, or odd beliefs. If delusions or hallucinations are present, they are not prominent and are not accompanied by strong affect. The course of the residual type may be time limited and represent a transition between a full-blown episode and complete remission. However, it may also be continuously present for many years, with or without acute exacerbation.

The DSM-IV criteria for residual type are as follows:

A. Absence of prominent delusions, hallucinations, disorganized speech, and grossly disorganized or catatonic behaviour.

B. There is continuing evidence of the disturbance, as indicated by the presence of negative symptoms or two or more symptoms listed in Criterion A for schizophrenia (Table 1.1), present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

Positive and negative symptoms

Positive symptoms or florid, productive or type I symptoms refer to the delusions, hallucinations, and bizarre or agitated behaviours which are associated with acute onset. The negative or defect, deficit or type II symptoms are used to describe the characteristics of affective blunting, poverty of speech and thought content, apathy, anhedonia and poor social functioning. These symptoms are sometimes associated with an insidious onset, and a chronic course but can also occur after positive symptoms have faded.

1.2.3 Other psychotic disorders resembling schizophrenia

The term "psychotic" has historically received a number of different definitions, none of which has achieved universal acceptance. The narrowest definition of psychotic is restricted to delusions or prominent hallucinations, with the hallucinations occurring in the absence of insight into their pathological nature.

In addition to schizophrenia, the following disorders are included in the same section in DSM-IV:

Schizophreniform disorder

Schizophreniform disorder is diagnosed when all the criteria for schizophrenia have been met except that the symptoms have been present for at least one month but less than six months.

Schizoaffective disorder

This diagnosis represents a syndrome when there is either a major depressive episode, a manic episode, or a mixed episode concurrent with the major symptoms of schizophrenia. The psychotic episode, i.e. delusions or hallucinations has to have persisted for at least two weeks in the absence of prominent mood symptoms. It may represent an overlap between the two major psychoses or a group of individuals exhibiting specific features of a schizoaffective psychosis.

Delusional disorder

Patients with this disorder experience nonbizarre delusions (i.e., involving situations that occur in every day life) for at least 1 month, have never met criterion A for schizophrenia (Table 1.1), and function reasonably well aside from the impact or ramifications of their delusions. If mood episodes occur concurrently with the delusions, their total duration is brief.

Brief psychotic disorder

Brief psychosis is diagnosed when schizophrenia like symptoms, either delusions, hallucinations, disorganized speech, or grossly disorganized or catatonic behaviours, have been present for at least 1 day and less than one month, with eventual complete recovery.

Shared psychotic disorder (Folie à Deux)

Patients with this disorder develop a delusion that is similar in content to the

already established delusion of another person with whom they have a close relationship.

Psychotic disorder due to a general medical condition

Patients with this disorder develop prominent delusions or hallucinations that are judged to be caused by a general medical condition and do not occur exclusively during the course of delirium or dementia.

Substance-induced psychotic disorder

The disorder includes prominent hallucinations or delusions associated with evidence that the symptoms developed within 1 month of significant substance intoxication or withdrawal, or is aetiologically related to medication use or toxin exposure. The common such substances include alcohol, amphetamines, cannabis, cocaine, hallucinogens, inhalants, opioids, phencyclidine, sedatives, hypnotics, and anxiolytics, etc.

1.3 Genetic factors of schizophrenia

Multiple research paradigms have provided evidence for a substantial genetic component in the aetiology of schizophrenia. These include family, twin, and adoption studies.

1.3.1 Family relative risk studies

Family studies of schizophrenia constitute one of the largest bodies of literature in psychiatry. In his earliest descriptions of dementia praecox, Kraepelin stressed the aetiologic importance of familial factors in this disorder (Kraepelin 1904). Generally the studies have observed the lifetime risk of the illness in the relatives of schizophrenics and compared this with the risk in control families and the general population. This method did not distinguish between genetic and environmental factors. However if a disease is non-familial then it is less likely that heritable

factors play a substantial role in its aetiology.

The first such study performed by Rudin in Munich (Rudin 1916) showed an increased incidence of schizophrenia in their relatives, this has been subsequently confirmed by many other workers (Reviewed by Gottesman and Shields, 1982). A valid criticism of these earlier studies was that they did not use standardised diagnostic criteria or controls and the probands as well as the relatives were not diagnosed blindly.

For a meaningful interpretation of family data the following methodological requirements should be used:

1. Structured interview schedules with explicit inclusion and exclusion criteria for psychiatric illness and good interrupter reliability.
2. Blind evaluation of family members with respect to diagnosis and kinship status.
3. The family study method (i.e., direct interviews).
4. Reliable operational criteria for the schizophrenia spectrum disorders.

One of the first such studies by Tsuang (1980) reported on the morbidity risks of schizophrenia and affective disorders among the first degree relatives of probands with schizophrenia, mania, depression and surgical conditions as a control group. Relatives of 200 schizophrenics, 100 manics, 225 depressives and 160 controls matched for sex, pay status, and age were personally interviewed some 30 to 40 years after the probands admission (so that most relatives had passed through the risk period for schizophrenia 15 to 45 years) using the Iowa structured psychiatric interview. Blind diagnosis of first degree relatives of the probands gave a morbidity risk for schizophrenia of 4.3%. This may be lower than expected since many of the relatives of the probands were parents and since schizophrenics have a reduced fertility the sample may contain many non-penetrant carriers. However the result was significantly higher than the control group.

Baron (1983) studied the familial relatedness of schizophrenia and schizotypal states using the method of parental mating types. Matings between two affected individuals should have a greater risk in the offspring than matings with one affected individual or two unaffected individuals. RDC and DSM-III criteria gave a morbidity risk for schizophrenia of 7.3% with one affected parent, and a risk of

14.5% for definite schizotypal personality disorder. This study indicates that schizotypal disorder may be genetically related to schizophrenia or at least that it occurs in the same families. However Baron noted that the siblings in the sample were not all through the age of risk for schizophrenia and this raises the possibility that some of the cases of schizotypal disorder represent preschizophrenic states and not a separate illness category.

Two larger studies (Guze, 1983; Baron et al., 1985a) also show significantly elevated risks for schizophrenia in relatives of probands. Guze carried out a 6-12 year follow up study of 500 schizophrenic probands and 1249 relatives using Feighner's classification. The overall risk in first degree relatives for definite and probable schizophrenia was 8.1%, significantly higher than the control group. Interestingly the sensitivity of these criteria at the start of the study was lower than at follow up because approximately one third of the cases only became evident at follow up. Many of these later cases received diagnosis of unipolar depression or schizophreniform disorder. The risks reported by other studies in other populations could be lower than the true value because longitudinal follow up information is often not collected.

In the study by Baron et al. (1985a) 750 first degree relatives of 90 chronic schizophrenics were personally interviewed. The control group was taken from randomly chosen acquaintances of the well siblings of the schizophrenic probands and matched for age, socioeconomic status and ethnicity. Schizophrenia was diagnosed according to the RDC since it has a more restrictive definition of chronic schizophrenia. All other diagnoses were according to DSM-III. The average risk to siblings and parents was 8.5 and 4.4% respectively. The difference was attributed to the reduced fertility of schizophrenics. The risks were significantly different to the risk in the control group. The study nullifies the argument of Pope (1982) that other types of psychiatric disorders may exaggerate the familiarity of schizophrenia since in this study the sample was based on chronic schizophrenia. The study of Guze (1983) showed no evidence of major depression in schizophrenic relatives and this also argues against the hypothesis of Pope (1982).

A study by Kendler et al. (1985) further resolved the controversy over the

familiarity of DSM-III schizophrenia. Personal interviews of 723 first degree relatives of 253 patients and 1056 first degree relatives of 261 surgical control patients were performed. In all of the analyses the risk for schizophrenia was significantly greater (18 times) in the relatives of schizophrenics than those of the controls. The risk of other non-affective psychotic disorders was also significantly higher in the schizophrenic group than in the controls.

Gershon (1988) studied a relatively small sample of 24 schizophrenic probands and 108 relatives, diagnosed according to RDC criteria. A significant increase in risk for schizophrenia compared to the control group was found even though the less sensitive telephone interview system was chosen for the data collection.

Family studies can also help clarify the familial relationship between schizophrenia and other psychiatric disorders. In the early history of psychiatric genetics the view was held that a single inherited predisposition to mental disease existed (Griesinger, 1861). In contrast Kraepelin (1904) articulated that the causes of different psychiatric disorders should be distinct. The data from Tsuang (1980) supported the distinction between schizophrenia and affective disorders as separate entities. The distinction between schizophrenia and mania alone was less clear cut. Similar results were demonstrated in Kendler's 1985 study in which schizophrenia and unipolar disorder were found to be independent and unrelated. The risk for bipolar disorder and affective disorders with psychotic symptoms was elevated in the relatives of schizophrenics but was not significantly different from the controls. One interpretation is that if affective disorder develops, a familial predisposition to schizophrenia may lower the threshold for the development of psychotic symptoms. Another interpretation is that the presence of atypical features could complicate diagnosis. Thirdly there may be assortative mating for schizophrenia and bipolar disorder.

The situation with schizoaffective disorder is less straightforward. Gershon (1988) demonstrated that there was no tendency for schizoaffective disorder to aggregate separately from schizophrenia. An increased incidence of bipolar disorder was found in the relatives of patients with schizoaffective disorder but not in the relatives of patients with schizophrenia. This leaves the conundrum as to whether

schizoaffective disorder is a bridge in a continuum of the major psychoses or a separate entity. Baron et al. (1982) attempted to solve the issue. He subdivided a group of schizoaffective disorder probands according to the predominance of affective or schizophrenic symptomatology according to the RDC and carried out a family study. This subdivision enhanced the familial resemblance between the schizoaffective affective type and affective disorder on the one hand and between the schizoaffective schizophrenic type and schizophrenia on the other hand.

In summary the results of these family studies using modern diagnostic criteria verified the original findings that schizophrenia is a familial disorder. They also indicate that the spectrum disorders are also familial and related to schizophrenia. Affective disorders tended to aggregate independently of schizophrenia, but the case of bipolar illness is less clear cut. This could be due to atypical features of psychosis and might become apparent at follow up. Schizoaffective disorder clearly segregates in both schizophrenic and bipolar affective disorder families. Whether it provides a link between the major psychosis is unclear. Separation of schizoaffective disorder into mainly affective or schizophrenic types (Baron, et al., 1982) provides evidence for a dichotomy of the psychoses.

These results have important implications for future familial genetic studies of segregation and linkage. The selection of disorders which are possibly genetically related to schizophrenia on the basis of family studies will reduce the effects of false positive and false negative cases in the analyses. In addition a careful choice of diagnostic criteria and interview methods must be made to clearly distinguish between disorders related and unrelated to schizophrenia.

1.3.2 Twin studies of schizophrenia

The twin method has been used to evaluate the role of genetic factors in a trait by the comparison of concordance rates for the disorder in monozygotic or identical twins and in same sex dizygotic or fraternal twins. Monozygotic twins have the same genotype while dizygotic twins, like full siblings, share on average 50% of their genes. The assumption often adopted in twin studies is that monozygotic and same sex dizygotic twins share environmental factors to approximately the same

extent. However models where there are unequal effects in twins have been used for behavioural traits. It seems likely that for such a severe disorder as schizophrenia then the equal environments assumption is valid. Therefore in the case of schizophrenia differences in concordance between the two twin types are likely to be due to the influence of genetic factors.

Despite this there have been several arguments to the use of twin studies for schizophrenia. One was that monozygotic twins may be more susceptible to schizophrenia because of their physical similarity and psychological relationship. However, there is no significant difference between the frequency of schizophrenia in monozygotic twins and that in the general population (Rosenthal, 1960). Another criticism of the twin approach is based on the observation that monozygotic twins share more of their social environment than do dizygotic twins, and the chance that this similarity in social environment rather than genetic similarity makes monozygotic twins more similar than dizygotic twins. Kendler (1983) in his thorough review argues against this conclusion. In an attempt to rule out the possibility of environmental bias in twin studies in schizophrenia, Kendler (1983) showed that monozygotic twins who were very physically similar were no more likely to be concordant for schizophrenia than twins who were relatively dissimilar. His results suggest that differential treatment by the social environment on the basis of physical similarity between twins is not likely to be a significant bias in twin studies of schizophrenia. Further clarification on this point came from a study by Gottesman (1982) who reported on 12 monozygotic twins who had been separated early in life and reared apart. Seven of these (58%) were concordant for schizophrenia and this has been interpreted as being highly suggestive of a genetic influence.

The evidence to date seems to validate the twin method as an approach to estimate the genetic contribution to schizophrenia. This evidence, as pointed out by Kendler (1983) is susceptible to a number of "reverse biases" which may decrease the difference in concordance in monozygotic and same sexed dizygotic twins. There are two main factors for this possible bias: assortive mating and the twin transfusion syndrome. Assortive mating has been shown to occur for a number of

mental illnesses (Slater, 1971). This would result in dizygotic twins sharing certain alleles more than predicted by random mating. This would increase the dizygotic twin concordance for a dominant disorder and the monozygotic twin concordance would remain the same, thereby decreasing the difference between the two. For a recessive disorder assortive mating might increase both monozygotic and dizygotic concordance over and above that expected from the population base rate.

Monozygotic twins can be either dichorionic or monochorionic, and in monochorionic twins sometimes differential transfusion of blood occurs between twins. This is called the twin transfusion syndrome which can sometimes result in large differences in the weight and size of monozygotic twins at birth. Obstetric complications may be of aetiologic importance in schizophrenia (Jacobsen, 1980; Owen, 1988). These are hypothesized to act as precipitating factors on a genetic susceptibility since no increase in the frequency of schizophrenia is recorded amongst monozygotic twins. It might be argued that twin transfusion could decrease the concordance for schizophrenia in monozygotic twins leading to an underestimate of the importance of genetic factors. In conclusion the evidence points to the fact that twin studies for schizophrenia are probably valid and other complicating factors may reduce the estimate of the importance of genetic factors.

A higher proband-wise concordance rate for schizophrenia in monozygotic twins than that in dizygotic twins would implicate genetic mechanisms in the aetiology of schizophrenia. Kendler (1983) summarized nine twin studies including 401 monozygotic and 478 dizygotic twins and reported concordance rates of 53 % and 15 %, respectively. Gottesman (1982) also reviewed 6 twin studies published during the past 25 years. The proband-wise concordance rates are 48 % for monozygotic twins and 17 % for dizygotic twins. All of these review studies showed that individuals who have a schizophrenic monozygotic twin are 3-4 times more likely to develop schizophrenia than those who have a schizophrenic dizygotic twin.

An extension of the twin method has been to investigate the frequency of schizophrenia in the offspring of schizophrenic monozygotic twins and their normal co-twins (Fischer, 1971; Gottesman and Shields, 1989). This would allow the comparison of the frequency of schizophrenia in a group where one of the parents

has schizophrenia with another group where one of the parents have the same genes as a schizophrenic person but no psychotic illness. One goal of this strategy is to try to differentiate between genetic factors that are necessary for the development of schizophrenia and other factors that may increase risk. Such factors could be triggers, exacerbators, or contributors to severity or poor outcome. Fischer's (1971) sample contained eight monozygotic pairs discordant for schizophrenia, with a total of 11 offspring from the probands and 28 offspring from the unaffected co-twins. The conclusion of this Danish study was that the frequency of schizophrenia and schizophrenia like psychosis in the children of schizophrenic and normal monozygotic twins was equal. The results were interpreted as demonstrating that genetic factors present equally in the schizophrenic and normal monozygotic twins were responsible for causing schizophrenia and that environmental factors associated with being reared by a schizophrenic parent had little influence. However, because of the limited number of individuals investigated, the results were not statistically significant and could have occurred by chance. At best they demonstrate a trend.

Kringlen (1989) analyzed offspring of discordant monozygotic twins from Norway and found no significant difference in the rate of DSM-III schizophrenia and schizotypal disorder in the offspring of the proband compared with their normal co-twins. However they did report a non-significant trend that schizophrenia spectrum disorders were less frequent in the offspring of non psychotic co-twins compared to that of the index twin. They argued that this might be evidence for the role of environmental factors modifying schizophrenia by the effect of a schizophrenic parent on a child. However this study also had a limited number of individuals and the findings could have arisen by chance. Another criticism of the Norway study was that only 40% of the offspring had gone through the risk period for schizophrenia (15-45 years).

Gottesman (1989) carried out a follow up study on Fischer's twin group 18 years after the original study in order to clarify the situation. All individuals were classified using DSM III-R criteria. They found a morbid risk for the schizophrenia and related disorders in the offspring of schizophrenic monozygotic twin and that of the normal co-twin to be 16.8% and 17.4% respectively. They also analyzed the

risks in the offspring of discordant dizygotic twins. The risks were 17.4% for the offspring of the schizophrenic twin and 2.1% for the normal co-twin. The risk for the offspring of the normal monozygotic co-twin compared to the normal dizygotic co-twins were found to be significantly different. They concluded that there was no support for the hypothesis that rearing by a schizophrenic parent is necessary or sufficient for causing schizophrenia in the offspring.

1.3.3 Adoption studies of schizophrenia

Adoption and cross fostering studies seek to further clarify the respective contributions of the genetic endowment and the environment because this method separates the effects of genes and the environment more clearly than in family risk or twin studies.

The most plausible non-genetic form of familial transmission for a behavioural syndrome like schizophrenia is "vertical cultural transmission" in which a particular trait is learned by offspring from their parents. Religious belief and political affiliation are probable examples. For schizophrenia pure cultural transmission would predict that the high risk for schizophrenia in the children of schizophrenic parents results from "learning" to be schizophrenic from their parents. Based on this hypothesis, children of schizophrenic biologic parents who have been reared by non-schizophrenic adoptive parents are supposed to have a low risk for schizophrenia, and children of a non-schizophrenic biologic parent reared by an adoptive schizophrenic parent are supposed to have a high risk for developing schizophrenia.

There are several types of adoption study designs, for example, adoptees method, adoptees' family method, and cross-fostering. The adoptees method is to study the adopted away children of schizophrenic biological parents. If the morbid risk in these adoptees is higher than that in adoptees born to unaffected biological parents, then a genetic component can be invoked. Heston (1966) compared 47 adopted children whose biological mothers were schizophrenic with a control group of 50 adopted children whose biological mothers were not schizophrenic. 11% of the children with schizophrenic biological mothers grew up to be schizophrenic and none of the children in the control group developed schizophrenic. Similar results

were also reported in Rosenthal et al.'s (1968) studies in which 7.7% of the adoptees with schizophrenic biological mothers developed schizophrenic and none of the children in the control group developed schizophrenic.

The adoptees' families method assesses the risk for schizophrenia between the biological and adoptive relatives of affected adoptees. If the morbid risk of the biological relatives of affected adoptees is greater than that of their adoptive relatives, then a genetic effect can be proposed. Kety et al. (1975; 1983) ascertained schizophrenic and control adoptees and compared the morbid risk for schizophrenia of their biological and adoptive relatives. He found that 6.4% of the biological relatives of the schizophrenic adoptees were also schizophrenic, compared with only 1.4% of their adoptive relatives were found to be schizophrenic.

The cross-fostering method is the study of adoptees whose biological parents are unaffected but the adoptive parents later became schizophrenic. Wender et al (1974; 1977) found no increased risk of schizophrenia in the adopted away offsprings of normal parents, who were subsequently raised by schizophrenic adoptive parents. This result suggested that the effects of family environment on the developing schizophrenia are rather small.

The adoption study results taken together suggest that simple cultural transmission is not likely to play a major aetiologic role in schizophrenia. The available evidence from the adoption studies indicates that schizophrenia is not "learned" from parents but genetic factors are the most influential cause of the familial transmission. These genetic factors are also important in the liability of several schizophrenic like syndromes including schizoaffective disorder, schizotypal disorder and paranoid personality disorder. Since the familial clustering of schizophrenia is an expression of shared genetic factors, the application of molecular genetic techniques to informative high-density pedigrees can be justified.

1.4 Genetic transmission models of schizophrenia

To test a particular genetic hypothesis in human populations the geneticist has

to fit probability models to family data: that is by comparing the observed proportion of affected siblings and offspring with the proportion expected according to a particular genetic hypothesis. This is referred to as segregation analysis. The main problems of such studies arise from the different methods of ascertaining families and affected individuals, pooling data from different families, incomplete ascertainment, inaccurate diagnosis, and genetic heterogeneity.

Statistical support for a specific genetic model can be considered as evidence in favour of an underlying genetic aetiology. This information would be invaluable in genetic linkage studies, because demonstration of a major gene effect would provide impetus for the linkage strategy and greater precision in the estimation of the genetic parameters for that analysis. Identification of the mode of inheritance could help to elucidate the genetic mechanisms which have maintained schizophrenia in the population despite its apparent selective disadvantage due to reduced fertility. Finally, a precise knowledge of genetic transmission would be useful in genetic counselling.

Genetic modelling of the transmission for complex disorders can take into account reduced penetrance, phenocopies and multifactorial-polygenic (MFP) background effects. Reduced penetrance is interpreted as the incomplete or absent manifestation of a disorder in individuals who have the disease genotype. For schizophrenia evidence of this is demonstrated from family studies and the finding of discordance in monozygotic twins. Phenocopies are those individuals who manifest the disorder even though they do not carry the disease genotype, possibly due to environmental or other genetic effects. MFP is interpreted as more than one gene of additive effect (equal weight) contributing to an individuals liability to the disorder acting jointly with environmental effects.

A further two factors that are important for the correct parameterisation of transmission models in schizophrenia are the diagnostic criteria being used and genetic heterogeneity. A too restrictive definition of schizophrenia would result in a large proportion of false negative cases, whilst a too inclusive definition would generate many false positive cases. As described above studies of twins have indicated that RDC and DSM-III criteria for schizophrenia provide an empirically

useful approach for genetic studies. However the genetic models available do not easily allow for heterogeneity of linkage. In this case, one must employ suitable statistical methods to detect the fact that single major gene effects in schizophrenia at several loci can operate independently to produce schizophrenia.

Segregation analysis of family data employs likelihood methods which permit comparisons of genetic models under certain assumptions. In practise conclusive evidence for one model cannot be found because the extent of underlying heterogeneity of gene effects is unknown. Likelihood refers to the probability of observing a given data set under a particular hypothesis. The values of the genetic parameters that maximize the likelihood are the maximum likelihood estimates of these parameters. The ratio of the maximum likelihood under the null hypothesis (the observed pattern occurring by chance) to the maximum value of the likelihood under a particular genetic model is termed the ratio criterion. Therefore the smaller the likelihood ratio the less likely the null hypothesis is true. The statistical analysis consists of rejecting the null hypothesis by the ratio being smaller than that of a certain test statistic (for example, a Chi-square value). If the underlying assumptions are varied, analyses may favour a number of models none of which can be conclusively proven. There are at least three types of genetic analyses which have been used for schizophrenia with family pedigree data. These are the single major locus (SML), multiple locus, and mixed models.

1.4.1 The single major locus (SML) model

This model is based on the assumption that the inheritance of a disorder is a consequence of a single locus with two alleles (A,a) (Morton, 1955; 1991). Three genotypes are distributed throughout the population AA and aa the homozygous states, and Aa the heterozygous. These genotypes are each given a mean on a liability scale and are represented by three normal distributions whose variances are determined by the amount of other non-allelic (genetic or environmental) variance. The peaks of each curve is the mean value for the population as a whole for each genotype. The penetrance of the genotypes is the measure of the area of the normal curves above a certain threshold value on the liability scale.

SML model postulates that alleles at a single locus are responsible for the transmission of schizophrenia. Other genes and environmental factors may only play minor roles in the expression of the disease or its age of onset. For non-Mendelian disorders the following modifiers may apply: 1) reduced penetrance of the pathogenic gene, 2) the existence of phenocopies, 3) the addition of an environmentally related liability-threshold construct. However, even with those modifications of the SML model, the schizophrenia morbid risk to monozygotic twins and the offspring of two schizophrenics still could not be correctly predicted by SML models (O'Rourke et al, 1982).

1.4.2 The multiple locus models

Multiple locus models assume that genes found at more than one locus with small, independent and additive effects are responsible for the familial aggregation of schizophrenia. These models can be further divided into two types according to the number of genes involved in the disease:

1.4.2.1 Multi-factorial polygenic (MFP) model

The multi-factorial polygenic (MFP) model proposes that a large, unspecified number of genes and environmental factors combine additively to cause the disease. Several studies using a prevalence analysis method found that MFP models can explain the transmission model of schizophrenia (Gottesman & Shields 1967; Hanson et al, 1977). McGue et al (1983; 1985) applied a path analytic model to family data on schizophrenia assuming MFP transmission and found a high heritability (0.67) along with a low common family environment factor (0.29) when a broad definition of schizophrenia was used.

1.4.2.2 Oligogenic model

The oligogenic model assumes that several genes may act additively, interactively or multiplicatively on the aetiology of illness. Risch (1990a) conducted a simulation study to figure out the most possible genetic inheritance model of schizophrenia and suggested that there are at least three common genes acting

multiplicatively on the risk of this illness. Recently this model has been regarded as the most plausible inheritance model for schizophrenia (Moises et al, 1995; Owen & Craddock, 1996; Sham, 1996).

1.4.3 Mixed models

The mixed models assume that the liability to develop a disorder can be due to a single major locus (SML) component, a multi-factorial polygenic (MFP) component, a common environmental effect, and/or a random residual environmental effect. The mixed model combines that of the SML with a polygene background and environmental effects split into common environment within sibship and random environment. The advantage of this model is that the relative contributions of a SML and MFP effects to the overall liability can be evaluated.

Risch & Baron (1984) reported a mixed model analysis of data on 79 schizophrenics and their families and concluded that although a MFP model also fits the data, these family data were consistent with a mixed model which had a recessive major gene, high gene frequency and a very low penetrance. However, subsequent segregation analysis attempting to test the mixed model has yielded inconclusive results (Vogler et al, 1990).

1.4.4 Other models

Other models include the two major locus theory which examines the inheritance of two separate loci that interact in the development of the disorder (Kidd, 1973; 1997) and a polygenic model with graduated gene effects to allow variable contributions to the liability from several loci (Matthysse, 1986).

1.4.5 Results from segregation analyses applied to schizophrenia

The general findings of a number of studies are given in the Table 1.2 which shows that there is considerable variation between studies. In summary seven out of a total of twelve SML analyses found evidence to support this model (see Table 1.2), whilst four out of five MFP analyses were in favour of that model (see Table 1.2). Two studies rejected both of these hypotheses as opposed to one study which

found an acceptable fit to both (Matthysse, 1976; Baron 1982; Kidd, 1973). Two studies using more complex analyses provided evidence for the two locus hypothesis however one of these investigations also could not reject the SML and four locus models (Debray, 1978 and 1979; Book, 1978). Only two studies have investigated the mixed model and both found the family data compatible with this hypothesis (Risch et al 1984). These conflicting results are likely to be a consequence of population differences due to the presence of multiple genetic subtypes, but diagnostic uncertainties and over simplifications of the true genetic model may also contribute.

Nearly all of the studies were based on average risk figures for different classes of relatives of the probands and did not use the information available by including the particular segregation pattern of the disorder within families. Five studies which did take into account pedigree structure (see Table 1.2) nevertheless produced conflicting results. One study (Risch et al 1984) provided statistical evidence for a major locus with a recessive mode of transmission, however the ascertainment of schizophrenic pedigrees might be said to have an inherent bias favouring this model because of the presence of reduced fertility resulting in the selection of families where the parents of schizophrenics are unaffected. Another potential pitfall is that only eight of the studies in the table tested different models simultaneously and support for one mode of inheritance can only be considered strong if all other types of inheritance have been excluded for the same data.

In general the analyses are inadequate to account for all the variables affecting the true mode of transmission. Important factors such as genetic heterogeneity are ignored, and interactions between genes or epistasis are not accounted for in the models. Furthermore assortive mating and reduced fertility which are both relevant to schizophrenia are not dealt with adequately, and the populations analyzed may have not been large enough to produce a statistically unequivocal result. The limitations of the analytical methodologies are further compounded by diagnostic uncertainties. Therefore the following requirements seem necessary for family studies and genetic modelling in schizophrenia. 1) Studies should be based on a well defined series of probands and families from within a single population in order to

alleviate the extent of heterogeneity in the sample studied. 2) Sampling bias should be reduced by using prospective proband identification. 3) Operationalized diagnostic criteria should be used to increase the reliability of psychiatric diagnosis and allow comparison between studies. 4) Personal interviews rather than the family history method should be used to reduce ascertainment biases particularly for the schizophrenia spectrum. 5) Blind diagnoses by the clinicians should be carried out. In Table 1.2 it is disappointing that only two studies have taken these obvious rules into account (Tsuang et al., 1982 and 1983; Baron et al 1982). It is also noteworthy that these two studies included the spectrum disorders. On the assumption that such disorders are truly manifestations of the same genetic aetiology as full blown schizophrenia, which the family and adoption studies seem to indicate, studies which do not include these cases as affected will have a number of false negatives.

In summary, classical segregation analysis to sort out these factors appears hopeless, because there are seldom enough family data to estimate the large number of unknown parameters required to accurately model the complex trait. Nevertheless linkage analysis is considered robust enough to detect a major or minor gene effects despite incorrect assumptions being incorporated in the linkage analysis (Clerget-Darpoux, 1991). The risk ratio, another method of genetic modelling, decreases with the degree of relationship between a proband and relatives, and the rate of decrease depends on the mode of inheritance underlying the trait. Risch (1990b) applied the risk ratio to prevalence figures for schizophrenia and demonstrated that they best fitted an epistatic model of two or three major loci. This supports the use of linkage analysis to find these genes.

Table 1.2 Summary of family studies on the genetic models of schizophrenia

Study	Sample	Origin	Finding
SML analyses			
Book et al 1953	Sibs/parents	Northern Sweden	SML
Slater 1958	Sibs/parents	Germany	SML
Garrone 1962	Sibs	Switzerland	SML

Heston 1970	MZ twins Sibs/offspring, parents	Europe/USA	SML
Elston et al 1971	MZ/DZ twins relatives	Germany/USA	SML
Slater et al 1971b	Sibs/offspring relatives	Europe	SML
Karlsson 1974	Pedigrees	Iceland	SML
Kay et al 1975	Sibs/parents	England	SML rejected
Elston et al 1978	Pedigrees	USA	SML rejected
Tsuang et al 1982	Sibs/parents	USA	SML rejected
O'Rourke et al 1982	MZ/DZ twins Sibs/parents, offspring	Europe	SML rejected
Risch et al 1984	Sibs/parents	USA	SML rejected
<u>MFP analyses</u>			
Gottesman 1967	MZ/DZ twins relatives	Europe	MFP
Rao et al 1981	MZ/DZ twins relatives	Europe	MFP
Tsuang et al 1983	Sibs/parents	USA	MFP rejected
Ungvari 1983	Sibs/parents	USSR	MFP
McGue et al 1983	MZ/DZ twins relatives	Europe	MFP
<u>Studies of both models</u>			
Kidd et al 1973	MZ/DZ twins relatives	Europe	SML and MFP
Matthysse et al 1976	MZ twins Sibs/offsring	Europe	SML/MFP rejected
Baron 1982b	Sibs/parents offspring	Germany	SML/MFP rejected

Two-locus model			
Elston et al 1977	Pedigrees	USA	rejected
Studies of multiple loci			
Debray 1978&79	Pedigrees	France	SML/2-locus/ 4-locus
Book et al 1978	Pedigrees	Sweden	2-locus
Mixed SML-polygenic model			
Carter et al 1980	Sibs/offspring	USA	SML and MFP
Risch et al 1984	Sibs/parents	USA	SML with polygenic effects MFP rejected

1.5 Genetic linkage studies of schizophrenia

1.5.1 Introduction to genetic linkage analysis

1.5.1.1 Genetic markers

Naturally occurring DNA sequence variation provides an abundant supply of genetic markers. The first DNA markers were called Restriction Fragment Length Polymorphisms (RFLPs). These are DNA variations that create or destroy a cleavage site for a specific restriction endonuclease, or alter the number of bases between restriction enzyme sites, causing a change in originally detected by Southern Blotting (Southern, 1975) but it is now more common to use Polymerase Chain Reaction (PCR, Mullis and Faloona 1987) based methods.

During the last ten years a new type of genetic marker, characterized by a variation in the number of repeats of DNA sequence has been recognized. The size of the repeated DNA varies from primate alphoid satellite DNA (> 1kb) (Tyler-Smith and Brown, 1987) through minisatellite DNA (Nakamura et al, 1987) to microsatellites which are 2, 3, 4 or 5 base pair sequences (Weber and May, 1989).

A microsatellite marker, which is also called a short tandem repeat polymorphism (STRP), is detectable by using the PCR to amplify the region of the genome containing the variable region and analysing the size of the product as a determinant by the number of repeats of the array (Litt, 1991). The most common type of microsatellite is $(CA)_n$ where n is ranging from 10 to 60 and its occurrence is, on average, every 30 kb throughout the genome (Stallings et al, 1991).

There are now a large number of DNA markers and the effect of using many markers has been investigated in relation to the statistical significance of the lod. Thompson (1984) demonstrated, for the use of multiple markers used in independent tests of segregation that the interpretation of lod scores for a particular disease locus, requires the same level of significance as a single test.

1.5.1.2 Linkage analysis

The aim of linkage studies is to examine the cosegregation of alleles at two loci, for example, two marker loci or one marker and one disease locus, in order to detect departure from independent assortment or to estimate the genetic distance between the two loci. This section focuses on marker-disease linkage.

Linkage analysis consists of identifying polymorphic genetic markers that are sufficiently close on a chromosome so that they are inherited together with the disease mutation locus from one generation to the next. In such cases the marker and disease are said to be linked. The distance between the disease gene and the marker locus can be calculated by observing the number of recombinations that occur between the two loci. Recombination is the rearrangement or crossing over of alleles following exchange of material between pairs of homologous chromosomes during meiosis. The closer the disease locus is to a marker, the less likely recombination is to occur. Recombination is measured by the recombination fraction theta ($0.0 < \text{theta} < 0.5$). This is the proportion of the number of recombinant offspring in the total number of offspring. A value of 0.5 of theta indicates random segregation of the disease and marker alleles, a value less than 0.5 indicates that linkage may be present.

The method adopted in determining linkage is the maximum likelihood estimate

of the recombination fraction based upon the relative probability (Pr) of having obtained the family. The latter is determined by calculating the probability of having obtained the various combinations of the particular traits under consideration on the assumption of there being no measurable linkage (theta =0.5) and comparing this with the probabilities based on a range of recombination fractions from 0.0 to 0.5.

$$Pr = \frac{P(\text{family, given theta}=0\text{-}0.5)}{P(\text{family, given theta}=0.5)}$$

For convenience, Pr is expressed as its logarithm. The Log10 of the relative probability is called the lod score (Morton, 1955). The maximum likelihood estimate of theta may be obtained by summing the lods for all the families studied against various values of theta, the recombination fraction value which corresponds to the maximum lod is taken as the best estimate. At any specific value of theta, a lod exceeding 3.0 is said to confirm linkage and a value less than -2.0 rejects linkage. A comprehensive account of linkage analysis has been given by Ott (1985). By considering the relative lengths of all 22 autosomes it has been calculated that the prior odds of linkage for any two genes (i.e. that two genes are syntonic) is 1:17.5 (Renwick, 1971). As a consequence a lod of 3 represents odds in favour of linkage of approximately 20:1 and not 1000:1.

A number of studies have used linkage methods in an attempt to detect genes of major effect in schizophrenia. These include linkage studies using classical markers and those using DNA markers.

1.5.2 Linkage studies using classical markers

In the early stage, all linkage studies of schizophrenia used conventional protein-based markers such as human leukocyte antigens (HLA), (Turner, 1979; McGuffin et al, 1983; Chadda et al, 1986; Andrew et al, 1987; Goldin et al, 1987; Campion et al, 1992), ABO blood types (Elston et al, 1971; Turner, 1979, McGuffin et al, 1983; Andrew et al, 1987), and immunoglobulin (Gm) (Elston et al,

1971). However, there was no replicated positive evidence for linkage or association of schizophrenia obtained from these classical protein polymorphisms.

1.5.3 Linkage studies using DNA markers

Two general strategies are commonly employed for identifying major genes in genetic disorders: 1) through testing regions with candidate genes, 2) systematic mapping of a disease locus using genetic markers evenly spaced to cover the entire human genome.

1.5.3.1 Linkage studies of schizophrenia based on cytogenetic abnormalities

The first positive linkage result of schizophrenia was with DNA markers for the chromosomal region 5q11-q13 (Sherrington et al, 1988). This followed the observation of a schizophrenic uncle and nephew with partial trisomy of a segment of the proximal long arm of chromosome 5 (Bassett et al, 1988). Unfortunately, subsequent investigators failed to replicate this linkage (Kennedy et al, 1988; Detera-Wadleigh et al, 1989; Hovatta et al, 1994). Indeed, a re-analysis of published data (McGuffin et al, 1990) suggested that the sole positive result would seem to be a chance finding and the disparate findings probably should not be interpreted as resulting from true linkage heterogeneity. Recently a significant linkage evidence near the 5q11-q13 region was found with D5S111 in a Puerto Rican pedigree (Silverman et al, 1996). More studies have to be done to confirm or replicate chromosome 5 linkage.

Several studies focused on chromosome 11q because balanced translocations of parts of chromosome 11q segregating with major psychotic illness in small pedigrees have been reported (Smith et al, 1989; Holland et al, 1990; St. Clair et al, 1990). Yet there is no positive linkage evidence to suggest the presence of schizophrenia susceptibility genes on chromosome 11q in other large multiply affected pedigrees (Nanko et al, 1992; Gill et al, 1993; Wang et al, 1993; Hovatta et al, 1994).

Another interesting region is the pseudoautosomal locus because schizophrenia occurs more frequently in the same sex of schizophrenic siblings than those of the opposite sex (Rosenthal, 1962) and also a high frequency of cytogenetic

abnormalities, for example, XXY and XXX, of the sex chromosomes may have been observed in schizophrenics (Crow, 1988). A number of linkage studies on the pseudoautosomal region have been reported (Collinge et al, 1991; DeLisi et al, 1991; d'Amato et al, 1992; 1994; Wang et al, 1994; Crow et al, 1994; Maier et al, 1995). None of these studies found conclusively positive linkage results with schizophrenia. Some other groups have also found chromosomal abnormalities on chromosome 2 and chromosome 9 (Genest et al, 1976; Nanko et al, 1993). No conclusive linkage evidence was found on these regions either (Aschauer et al, 1993).

1.5.3.2 Linkage studies of schizophrenia based on candidate genes

There are many published linkage studies of schizophrenia based on the candidate gene approach, i.e. genes encoding for neurotransmitter receptors or neurotransmitter enzymes (see section 1.6.1) that are thought to be involved in the aetiology of schizophrenia. These candidate gene studies included the five dopamine receptor genes (Mioses et al, 1991; Su et al, 1993; Weise et al, 1993; Barr et al, 1993; Coon et al, 1993; Jensen et al, 1993; Campion et al, 1994; Hovatta et al, 1994; Macciardi et al, 1994; Maier at al, 1994; Nanko et al, 1994; Ravindranathan et al, 1994; Sabate et al, 1994; Kalsi et al, 1995a; 1996a), dopamine transporter gene (Byerley et al, 1993; Persico et al, 1995), $GABA_A$ receptor gene (Hovatta et al, 1994; Byerley et al, 1995), glutamate receptor genes (Pariseau et al, 1994), serotonin receptor genes (Hallmayer et al, 1992; Hovatta et al, 1994) and so on. None of these studies have provided conclusive evidence for a major gene for schizophrenia (Kendler and Diehl 1993).

1.5.3.3 Linkage studies of schizophrenia using genome scan approach

Because of the uncertain pathogenesis of schizophrenia, an alternative is to conduct a systematic search using evenly spaced markers at about 10 cM intervals in order to identify major genes for schizophrenia. This approach became feasible in the light of recent advances in molecular technology and the large number of available polymorphic markers covering the human genome. Several large

multicentre collaborative projects have been launched all over the world and many systematic genome studies using larger collections of multiplex affected pedigrees and highly informative markers have been published. Several studies using this approach have identified several "hot spots" for schizophrenia such as chromosome 22q12-q13 (Coon et al, 1994; Pulver et al, 1994a,b; Vallada et al, 1995; Kalsi et al., 1995b; Lasseter et al, 1995; Schizophrenia Collaborative Linkage Group (Chromosome 22), 1996), chromosome 6p24-p22 (Wang et al, 1995; Straub et al, 1995; Schwab et al, 1995; Antonarakis et al, 1995; Moises et al, 1995; Schizophrenia Linkage Collaborative Group for Chromosomes 3, 6 and 8, 1996), chromosome 3p (Pulver et al, 1995; Schizophrenia Linkage Collaborative Group for Chromosomes 3, 6 and 8, 1996), and chromosome 8p (Pulver et al, 1995). Apparent confirmation of these findings has been published for 8p by Kendler et al. (1996) and other work leads to support linkage of schizophrenia to this locus (Kalsi et al., 1996b). Chromosome 6p linkage , likewise, has resulted in the finding of several non-parametric linkage analyses which are nominally statistically significant.

1.6 Vulnerability traits of schizophrenia

Vulnerability traits are potential sources of aetiological information underlying a disorder. Such traits are biological characteristics that are correlated with the genetic susceptibility to the disorder and are assumed to be a part of the pathway from the genotype to phenotype. Baron (1986) has reviewed the uses and limitations of vulnerability traits in relation to schizophrenia. Current findings for a number of susceptibility traits of schizophrenia will be summarised as follows:

1.6.1 Neurotransmitters/ neurotransmitter receptors/ neurotransmitter enzymes

A neurotransmitter is defined as a chemical that is synthesized in a neuron, is released by that neuron in response to electrical impulses, and acts on other neurons to alter their electrical properties. The types of molecules that mammalian neurons use as neurotransmitters include amino acids, monoamines, acetylcholine, peptides,

and a few small molecules such as nitric oxide, purines, etc. The most prevalent neurotransmitter in the central nervous system (CNS) are the excitatory amino acid glutamate (and possibly aspartate) and the inhibitory amino acids τ-amino butyric acid (GABA) and glycine. It has been estimated that these amino acids are utilized as neurotransmitters by 75%-90% of all of the neurons in the brain. It has become apparent in recent years that individual neurons frequently utilize more than one neurotransmitter for synaptic transmission. This finding contradicts what had been called Dale's law: one neuron, one transmitter. Indeed, there are many examples of the colocalization of multiple neurotransmitters within a single neuron. In most cases, neurons contain an amino acid, a monoamine, or acetylcholine plus one or more neuropeptides. Colocalized neurotransmitters within the same cell may be packaged in the same vesicles and released together or more often in separate vesicles and released from the same nerve terminal under different conditions (Hyman and Nestler, 1993).

Neurotransmitter receptors are proteins that mediate the actions of specific neurotransmitters on target neurons. They are found on the plasma membrane of dendrites, cell bodies, and/or axon terminals of neurons. Neurotransmitters bind to specific sites on receptor proteins. Such binding leads to alterations in the physiological properties of the receptors that result in transduction of the extracellular signal into an intracellular signal which leads, in turn, to alterations in the functional state of the target neurons.

It is not surprising that almost all of the major neurotransmitters, for example, catecholamines, indolamines, glutamate, GABA, opioids and other neuropeptides, etc., and their receptors have been suggested to have direct or indirect aetiological roles in the pathophysiology of schizophrenia. Among these, the dopaminergic system is one of the most predominant targets in the search of the aetiology of schizophrenia. Behavioural experiments in animals and receptor binding studies in vitro show that the major shared property of all commonly used antipsychotic drugs is their action as dopamine receptor antagonists. However, the so-called "atypical antipsychotics" such as clozapine, which is a relatively weak D_2 receptor antagonist, have been found to interact with many other neurotransmitter receptors, including

D_1, D_3, D_4, 5-HT_{2C}, 5-HT_2, etc. The observation that in the clinical use of antipsychotic drugs maximum clinical efficacy is found after a period of weeks regardless of immediate antagonism of dopamine receptors has conceptualized that a molecular mechanism and a multiple-neurotransmitter/neuromodulator interaction that produce as yet unknown chronic adaptations in brain function are the actual proximate causes of therapeutic improvement.

Neurotransmitter enzymes have a role in the metabolism of these neurogenic chemicals and therefore are thought to be involved in the pathophysiology of schizophrenia. A few examples are platelet monoamine oxidase (MAO), plasma amine oxidase (PAO), ~~erythrocyte~~ catechol-o-methyl transferase (COMT) and plasma dopamine-beta-hydroxylase (DBH). A proportion of the activity level of these enzymes are under the control of major autosomal genes and consequently they are heritable and stable. Chronic schizophrenia has been associated with reduced activities of both MAO and PAO (Baron et al., 1984a,b; 1985), whereas the data for COMT and DBH is contradictory (Baron et al., 1980; 1984a). Unfortunately there is little systematic family segregation data to make the apparent association of the enzyme activity with schizophrenia more obvious. Two studies of MAO (Wetterberg, 1979; Baron et al., 1984b) did find a significant association of reduced activity with ill relatives compared with well relatives of schizophrenic probands. However there are certain limitations to the usefulness of MAO as a trait marker. For example there is a considerable overlap of the activity levels between affecteds and unaffecteds, MAO activity may be influenced by neuroleptics and there is a high frequency of the genotype for low level activity of MAO in the general population. These considerations limit the scope for MAO as a major trait marker for schizophrenia. Similar findings and caveats also apply to PAO (Baron et al., 1985b). Studies with COMT showed no familial relation with schizophrenia (Baron et al., 1984a) but for DBH there is some inconclusive evidence for reduced levels of activity in schizophrenics with a family history (Wetterberg, 1979; Baron et al., 1980). Despite considerable efforts in the past these enzymes have yet to be implicated as useful vulnerability traits.

1.6.2 The immunologic and viral concept of schizophrenia

Since the last century, possible roles of viral infection in predisposition to schizophrenia have been suspected. Post-viral encephalitic conditions with symptoms resembling those of schizophrenia have been reported after known infections with influenza, mononucleosis, Epstein-Barr virus, and currently, human immunodeficiency virus (HIV), etc. The possibility of a neurotropic slow virus, which acts on individuals with specific immune deficiencies, has also been proposed.

This has been widely explored with inconsistent and conflicting findings (DeLisi 1984) and has included proposed pathological mechanisms such as elevated levels of serum immunoglobulins and interferon in cerebrospinal fluid of schizophrenics, altered states of the lymphoid system, and production of brain autoantibodies. The binding of serum globulin substance by human brain (a putative measure of brain autoantibodies) was found to be associated with schizophrenics. However high brain serum binding level differences between ill and well relatives of probands failed to reach statistical significance (Baron, 1977).

1.6.3 Neurophysiologic traits

Neurophysiologic studies of schizophrenia focus upon the identification of both trait-specific and state-dependent markers for schizophrenia and the significance of these physiologic mechanisms once they are identified. Smooth pursuit eye movements (SPEM), which are sometimes called "eye tracking", are the most well-established traits associated with schizophrenia.

Eye tracking is a heritable and stable characteristic. It is a potentially useful susceptibility trait since smooth pursuit eye movement (SPEM) dysfunctions are found in schizophrenics. SPEM is the slow-tracking lateral eye movement seen when an individual watches a swinging pendulum. Normally, the eyes move in a smooth back and forth sinusoidal pattern, but in some schizophrenics and other psychotic patients, this smooth pursuit is interrupted by multiple arrests in which the eye comes to a complete stop, resulting in an irregular pattern. The disruptions consist of a larger than expected number of saccadic intrusions and of saccadic smooth pursuit tracking that include "square wave jerks" during pursuit movements

and during fixation eye movements. Investigations have estimated that 50% to 85% of schizophrenic patients and 40% of manic depressives have these dysfunctions, whilst the population prevalence is only 8% (Holzman, 1984). Holzman also demonstrated a significant increase of eye tracking dysfunctions in the parents of schizophrenic probands (34% compared with only 10% for manic depressive parents). The eye tracking dysfunction in manic depressives however may be a consequence of lithium treatment to some extent. The first degree relatives of schizophrenic probands tend to have abnormal smooth pursuit eye tracking even if the proband's smooth pursuit is normal (Matthysse, 1986). To account for this finding Matthysse proposed that schizophrenia and disturbed eye tracking are independent expressions of an underlying latent trait which is genetically transmitted. Holzman (1988) later studied the prevalence of both schizophrenia and abnormal eye tracking in the first degree relatives of monozygotic and dizygotic twins discordant for schizophrenia and showed that the transmission of schizophrenia can be accounted for by a single dominant gene when the illness is considered together with abnormal eye tracking. However there are several important caveats of this study. In particular the data was only compared with the one model of transmission, and that model is probably oversimplified. The actual mode of inheritance of the eye tracking dysfunctions is still not clearly understood. Despite these limitations the latent trait model has considerable heuristic value and deserves further investigation before it can be considered as a model for genetic linkage studies.

Another approach to the study of cognitive deficit in schizophrenia has been the analysis of long-latency auditory event-related potentials (ERPs) which deal with the temporal dimension of specific electric events in the brain measured by monitoring specific electroencephalogram (EEG) leads. In particular the P300 (P3), the long-latency "cognitive" potential which occurs 300 milliseconds or so post stimulus when the subject is attending to an event that is unexpected. Abnormalities of P300 latency (increased) and amplitudes (reduced) have been found in schizophrenics (Blackwood, 1987; 1991; Romani, 1987). However the same abnormalities are known to occur in Alzheimer's disease, borderline/ schizotypal personality disorders and in other psychiatric and neurological disorders (Blackwood et al., 1986; 1990).

It is of interest that in multi-affected pedigrees a number of unaffected family members also have the abnormalities (Blackwood et al., 1990; 1991), and in some cases they have become affected at follow-up (Blackwood et al., 1991). Though these results are promising, in that a subtype of schizophrenia may be identified by ERPs abnormalities, further investigation is required and the mode of inheritance for this trait needs to be addressed.

1.6.4 Psychological traits

A set of psychological tests known as the information overload tasks (IOT) have been shown to be deficient in schizophrenic patients and in populations at genetic risk for schizophrenia (Cornblatt, 1985). These deficiencies do show some state independence however they cannot yet be considered as definite vulnerability traits because evidence for genetic transmission has not been reported.

1.6.5 Neuroanatomic pathology

The introduction of computed tomography (CT) offered the opportunity to study brain structure in living subjects. Over 90 investigations of brain structure in schizophrenics have been published (Shelton and Weinberger 1986). The general findings were enlargement of the lateral ventricles, enlargement of the third ventricle, atrophy of the frontal lobe, atrophy of the cerebellar vermis, abnormalities of brain density and reverse cerebral asymmetry in schizophrenics compared with normal controls. Further studies using the more sensitive magnetic resonance imaging (MRI) demonstrated that the area of the third ventricle in its most anterior coronal slice was increased by 73% in schizophrenic subjects and lateral ventricular volume was increased by 62% compared to normal controls (Kelsoe, 1988) and a reduction in the overall volume of cortical grey matter and of limbic structures such as the hippocampus (Zipursky, 1992; Harvey, 1993). These results have given rise to the hypotheses that schizophrenia is a neurodevelopmental disorder (Weinberger 1987). It has been demonstrated that cerebral ventricular volume is familial and probably due to a genetic effect (Reveley, 1984; DeLisi, 1986). Weinberger (1984) examined pairs of siblings and found that the schizophrenic sibling had a greater

ventricular size than the normal sibling whilst DeLisi (1986) demonstrated that change in ventricular volume was associated with the diagnosis of schizophrenia within families. Nevertheless further study is required because in a group of schizophrenics of twin birth no evidence of ventricular enlargement was found where there was a family history of schizophrenia (Reveley, 1984). However cerebral ventricular size was significantly increased in sporadic cases of schizophrenia associated with birth complications (Owen, 1988). Although an attractive hypothesis has been proposed (Lewis, 1987) that a distinction can be made between familial and sporadic forms of schizophrenia, and that this is reflected in brain appearance (with scan abnormalities occurring more frequently in the sporadic cases), it has been subjected to theoretical criticism and fails to completely account for the reports of brain scan abnormalities in apparently familial schizophrenia (Pearlson, 1985; Nasrallah, 1983). The original hypothesis that ventricular enlargement was largely confined to those patients with no evident family history is now probably untrue (Lewis, 1990). In a study of two British families (Gurling, 1992) one family showed a significant co-segregation between schizophrenia and multiple focal white matter high signal lesions.

In summary, recent neuroanatomical findings in schizophrenia indicate 1) that a fixed "lesion" is probably present from early in life, with most evidence implicating specific temporal lobe pathology, 2) that prefrontal, and possibly medial temporal, physiological dysfunction is an active process in the illness, and 3) that dopaminergic and perhaps other neurotransmitter systems appear to mediate clinical expression of the illness. A genetic liability clearly appears to facilitate the phenotypic expression of schizophrenia through an as-yet-unidentified mechanisms (Wolf et al., 1993).

In conclusion vulnerability traits provide a useful avenue of investigation but their use in determining underlying genetic aetiology is restricted because there has never been any strong evidence for their genetic involvement and because, in most cases, the genetics of the traits themselves are unclear. The most promising avenues of the search for an endophenotype appear to be brain morphology, SPEM dysfunctions and abnormalities of ERPs. The use of genetic linkage markers still

provides the most robust investigation into the genetic aetiology of schizophrenia. The true relationship between the genetics of schizophrenia and these endophenotypes will probably become known when a genetic subtype of schizophrenia has been identified through linkage studies.

CHAPTER 2: THE GLUTAMATERGIC DYSFUNCTION HYPOTHESIS FOR SCHIZOPHRENIA

2.1 Introduction to glutamatergic systems

L-Glutamate is the predominant excitatory neurotransmitter in vertebrate nervous systems. For example, the pyramidal cells of the cerebral cortex, the granule cells of the hippocampus, and the excitatory thalamocortical pathways appear to be glutamatergic (Robinson et al., 1987). Glutamate receptors are found throughout the mammalian brain. The longest-known and best-studied glutamate receptors are ligand-gated ion channels, also called ionotropic glutamate receptors, which are permeable to cations. Receptors for glutamate have been implicated in neuronal plasticity and higher neuronal functions such as memory and learning (Collingridge and Singer, 1990; Nicoll et al., 1988). Of particular medical interest is their role in acute neuronal cell death following hypoxia, hypoglycemia, ischaemia or epilepsy (Choi, 1988). Although the exact cellular mechanism leading to cell death still remains to be elucidated there is no doubt about the critical role that calcium ion plays in several settings which eventually lead to neuronal cell death.

2.1.1 Subtypes of glutamate receptors

Glutamate receptors have traditionally been classified into three broad subtypes based upon pharmacological and electrophysiological data: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and kainate (KA) receptors. Recently, however, a family of G-protein-coupled glutamate receptors, which are also called metabotropic glutamate receptors was identified (Hollmann and Heinemann, 1994; Seeburg, 1993). In this thesis, I only focus on the ionotropic glutamate receptors.

NMDA selectively gates a channel with comparatively much slower kinetics and high Ca^{2+} permeability. The functional aspects of NMDA receptors are collectively determined by these unique properties together with a voltage-dependent block by Mg^{2+}, a requirement for glycine as co-agonist, and a large channel conductance. AMPA activates channels with fast kinetics, i.e. onset, offset and desensitization

time-course are in the order of milliseconds, and these channels are characterized by very low Ca^{2+} permeability in most neurons. The potent neurotoxin kainate generates currents with different properties in distinct receptor channels: through AMPA receptors, kainate activates a current that persists in the continued presence of this agonist. However, through high-affinity kainate receptors, kainate activates fast desensitizing currents. (Seeburg, 1993)

2.1.2 Neurophysiological functions of glutamate receptors

The neurophysiological functions of these three channel classes (i.e., NMDA, AMPA, and kainate) can be summarized as follows: In NMDA receptor channels, Ca^{2+} flux is an important consequence of glutamate activation. Excessive NMDA receptor activity and concomitant Ca^{2+} influx can cause neuronal death. An important restriction to Ca^{2+} influx through the NMDA receptors is provided by the voltage-dependent Mg^{2+} block which curtails transmitter-evoked ion conductance at the negative resting membrane potential. Presynaptically released glutamate cannot activate any significant ion flow through the channel unless the postsynaptic membrane is sufficiently depolarized to remove the Mg^{2+} blockade of the channel.

AMPA receptors mediate the majority of all fast excitatory neurotransmission which can be rendered by their rapid kinetics. The generally low Ca^{2+} permeability of AMPA receptors indicates that it does not carry sufficient Ca^{2+} into cells to initiate a sequential intracellular biochemical processes triggered by an increase in intracellular Ca^{2+} levels when these AMPA receptor channels are activated by glutamate.

The function of high-affinity kainate receptors is not yet well understood. The natural agonist glutamate and the neurotoxin kainate both activate rapidly desensitizing currents in these receptors. However, such currents can be only detected in sensory ganglia but not so far in central neurons. The possible explanations would be: 1) Kainate also produces large, nonsensitizing currents via the AMPA receptors which are abundant in central neurons and may overshadow fast desensitizing currents from the less abundant kainate receptors. 2) Kainate receptors are localized only in dendrites and synapses but not on the cell soma where most patch recordings have been performed. 3) Kainate receptors might be located

presynaptically and function as autoreceptors (Seeburg, 1993).

2.2 Molecular biology of glutamate receptors

The modern classification of glutamate receptors (GluR) has been brought about by the cloning, sequencing and functional expression of their genes. Expression cloning in the Xenopus oocyte has led to the molecular characterization of two ionotropic GluR receptor subunits which are AMPA receptor subunits (Hollmann et al., 1989) and the principal NMDA receptor subunits (Moriyoshi, et al., 1991). Additional cDNAs encoding GluR subunits were obtained by homology screening and by PCR-based strategies. Up to now, at least twenty-eight recombinant glutamate receptor cDNAs plus a considerable number of splice variants thereof have been cloned (Hollmann and Heinemann, 1994) and most have been functionally analysed. The newly characterized nucleotide sequences have served as probes for investigating the spatial and temporal expression patterns of these cognate mRNAs in rodent brain. The combined sets of data from ligand binding, in situ hybridization and biophysical analysis have suggested that the molecular properties of the major GluRs are beginning to be understood.

2.2.1 NMDA receptors

The first NMDA subunit to be cloned was the NMDAR1 subunit from the rat (Moriyoshi et al, 1991). Subsequently cloning through hybridization with oligonucleotides based on sequences homologous to the transmembrane regions of NMDAR1 gene led to the isolation of three structurally related rat NMDA receptor subunits, NMDAR2A, NMDAR2B, NMDAR2C (Monyer et al., 1992) and finally a fourth subunit (NMDAR2D) was cloned (Ishii et al, 1993).

In molecular terms, the NR1 subunit is only distantly related to the NR2 subunits because the two subunit types share a mere 18% sequence identity. However, in spite of their substantial primary sequence divergence, NR1 and NR2 subunits contain an identical functional determinant in their putative channel-forming region, transmembrane II (TM II). This determinant, an asparagine (N) residue, is

located at a position occupied by glutamine or arginine in the homologous TM II sequence of AMPA and kanaite receptor subunits. The N position in NMDA receptor subunits mediates the high Ca^{2+} permeability and the voltage-sensitive Mg^{2+} block of the channel. Replacing the asparagine (N) by glutamine (Q) in NR1 leads to a decrease in Ca^{2+} permeability but does not increase the low Mg^{2+} permeability of the heteromeric channel. The same substitution in the NR2 subunit increases the Mg^{2+} permeability but fails to affect the Ca^{2+} permeability. This indicates that the N position of NR1 and NR2 subunits contributes unequally to the functional aspects of the heteromeric channel. As mentioned in the next section, AMPA and NMDA receptors contain common structural motifs in their TM II regions that are responsible for some of their divalent ion selectivity and conductance properties.

Heteromeric NMDA receptors are generated by assembly of the NMDAR1 subunit with specific members of the NMDAR2 subunit family which yielded about 100 times larger glutamate induced currents compared with homomeric NR1 expression. As for native NMDA receptors, these recombinant receptors require the presence of glycine as co-agonist. These heteromeric NR1-NR2 receptors can be efficiently gated by NMDA but not by AMPA or kainate.

In situ hybridization (Monyer et al, 1992; Ishii et al., 1993) shows a different expression pattern for the individual subunits. The NMDAR1 subunit is expressed ubiquitously in brain and is required for the normal function of the NMDA ionophore (Moriyoshi et al., 1991). NMDAR2 subunits are more discretely localized and appear to serve a modulatory role in NMDA channel function (Ishii et al., 1993). This implies in vivo subtypes of NMDA receptors. NR2A subunit mRNAs show the widest distribution within the NR2 family. NR2B mRNA shows a wide but more restricted distribution in its expression pattern. Its expression is prominent in most of the telencephalic and thalamic regions but low in the hypothalamus, cerebellum, and lower brain stem regions. NR2C message is found mainly in the cerebellum. Moderate expression of this mRNA is also observed in the olfactory bulb, some of the thalamic nuclei, pontine and vestibular nuclei. NR2D mRNA is mainly expressed in the diencephalic and lower brain stem regions. Weaker signals were found in the cerebral cortical regions and the granular layer of the cerebellum.

These NMDA receptor subtypes with their distinct physiological properties may produce synaptic plasticity by expression in specific cells.

2.2.2 AMPA receptors

Following the first isolation of a cDNA clone coding for a subunit of a glutamate gated ion channel (Hollmann et al, 1989), three closely related genes were identified. They are either referred to as GluR A-D (Keinanen et al., 1990) or GluR 1-4 (Boulter et al., 1990) subunits. These subunits are approximately 900 amino acids in length and occur in two major forms with respect to an alternatively spliced exonic sequence of 38 residues which precedes the last putative transmembrane region (TM IV). The two forms, named 'flip' and 'flop', of these splice variants in the N-terminal TM IV region are encoded by two different exons that are used in a mutually exclusive manner (Sommer et al., 1990) and display different expression profiles in the mature and developing brain. The prenatal brain expresses mostly 'flip' forms of GluR 1-4, and the expression of these forms persists throughout life. The 'flop' forms appear only from the early postnatal stage onwards and are co-expressed with 'flip' forms in many cells. In AMPA receptors of native and recombinant configurations, glutamate elicits a current response consisting of a fast desensitizing component and a steady-state component. Precocious receptors containing 'flip' modules appear to be characterized by slower desensitization kinetics than adult receptors that contain both 'flip' and 'flop' modules. These differing desensitization kinetics affect the ratio of peak vs. steady-state components of glutamate-activated currents in AMPA receptor channels. The steady-state component is virtually absent in channels assembled from subunits in their 'flop' configuration.

The four different subunits impart electrophysiological properties on the expressed recombinant receptors. The presence of GluR2 (GluR B) subunits in heteromeric channels determines the I-V characteristics in a dominant fashion: The I-V curve of receptors containing the GluR2 subunit is almost linear whereas receptor channels without the GluR2 subunit have a doubly rectifying I-V curve. Based on the information from site directed mutagenesis studies, the difference between GluR2 and other non-GluR2 AMPA receptors is located at the C-terminal

end of the TM II segment where the arginine (R) residue was found in the GluR2 subunit in contrast to the glutamine (Q) in the other AMPA receptors (Sommer, et al., 1991).

The more important characteristics than their distinct I-V relationships are the marked differences in their Ca^{2+} permeabilities. Channels without a GluR2 subunit show a significant permeability for Ca^{2+}. However, the presence of a GluR2 subunit renders these channels virtually impermeable to Ca^{2+}. The mechanism of the control of the Ca^{2+} permeabilities has been identified as the above-mentioned Q/R site where the presence of an R is responsible for the Ca^{2+} impermeability. The expression of the GluR2 gene is tightly controlled which indicates that brain cells may regulate the amount of Ca^{2+} passing through AMPA receptor channels by controlling the level of GluR2 gene expression.

Analysis of both rat and human genomic DNA sequences of these four AMPA receptors, GluR1, 2, 3, 4, revealed more interesting findings (Sommer et al., 1991; Lomeli et al., 1994, Paschen et al., 1994): Genomic DNA sequences encoding this particular channel segment of all four GluR1-4 subunits harbor a glutamine (Q) codon (CAG), even though an arginine (R) codon (CGG) is found in the cDNA encoding the GluR2 subunit. This process of post-transcriptional A --> G conversion has been identified as an RNA editing mechanism mediated by a double-stranded RNA-specific adenosine deaminase (Yang et al., 1995; Reuter, et al., 1995). Intron 11, which is located immediately downstream of the edited position (Q/R site), is essential for accurate and efficient RNA editing in GluR2. This intron contains a 10 nucleotide region that is complementary to the exonic sequence immediately surrounding the Q/R site. It has been proposed that this editing site complementary sequence (ECS), as well as an imperfect inverted repeat, contributes to the formation of an RNA duplex within the GluR2 primary RNA transcript (pre-mRNA) that is critical for editing.

2.2.3 Kainate receptors

High-affinity kainate receptors can be generated in vitro from subunits GluR5, GluR6, GluR7 and KA1 or KA2. In situ hybridization studies in rat brain showed that these five subunits form a complex mosaic of expression which indicates that

kainate receptors may be involved in all central neuronal circuits of the brain (Seeburg, 1993). Agonist-elicited current responses are observed in homomeric configurations of GluR5 and GluR6 but not GluR7 subunits. In vitro, KA1 or KA2 expression alone does not generate functional channels (Werner et al., 1991). However, when combined with either GluR5 or GluR6, new properties emerge in comparison with homomeric GluR5 and GluR6 receptors: Compared with homomeric GluR5 receptors, GluR5/KA2 channels show more rapid desensitization kinetics and display a different I-V relationship. GluR6/KA2 channels can be gated by AMPA which fails to activate homomeric GluR6 receptors.

As for the GluR2 AMPA receptor, both GluR5 and GluR6 but not GluR7, KA1 or KA2 occur in two forms with respect to the amino acid residue occupying the Q/R site in TM II through post-transcriptional RNA editing (Sommer et al., 1991; Lomeli et al., 1994, Paschen et al., 1994). GluR6 has two additional RNA editing positions in TM I where isoleucine is converted to valine (I --> V) and tyrosine to cysteine (Y --> C) respectively. PCR analysis has shown that 65% of GluR6 in rat brain are fully edited in TM I and TM II (i.e. V, C; R) while the unedited, genomically encoded GluR6 (I, Y; Q) subunit constitutes only 10%. The remaining 25% of RNA consists of combined edited/unedited subunits in these three potential editing sites.

It appears that only when the TM I positions are edited does the TM II Q/R site influence Ca^{2+} permeability in GluR6. However, in contrast to the GluR2 AMPA receptor, edited GluR6 (R) channels show a higher Ca^{2+} permeability than GluR6 (Q). RNA editing of GluR subunits represents the first known example of editing of neuronal RNA and adds another mechanism to generate receptor heterogeneity of functional importance.

2.3 Existing evidence suggesting the involvement of the glutamatergic system in schizophrenia

For over the past two decades the major explanatory hypothesis for the pathophysiology of schizophrenia has centred on the mechanism of antipsychotic

effects of neuroleptics in terms of blocking D_2 dopamine receptors (Seeman, 1987, 1989; Peroutka, et al, 1980). The "dopamine hypothesis" mentions that the clinical potency of neuroleptics correlates very well with affinity of these drugs for the D_2 receptors, and that stimulants, which enhance central dopaminergic neurotransmission, can produce the positive symptoms of schizophrenia such as delusions, hallucinations, and thought disorder. Postmortem as well as in vivo imaging studies (Wong et al., 1986) have provided reasonably compelling evidence that dysfunction of dopaminergic neurotransmission is an important element of this disorder. However, although paradoxical dopaminergic hypofunction might contribute to negative symptoms, dopaminergic dysfunction would appear to account primarily for positive symptoms (Carlsson et al., 1990). Furthermore, the fact that neuroleptics may take weeks to produce their full effect suggests that their mechanism of action is indirect, requiring neuronal adaption beyond more D_2 receptor blockade (Hyman et al., 1996). It would be even more difficult to rationalize dopaminergic dysfunction as a complete explanation of cognitive deficits, corticolimbic atrophy, and a possible deteriorating course.

Fundamental research into the dual role of glutamate as the major excitatory neurotransmitter in the brain and as the proximate cause of neuronal degeneration has led to new insights into the pathophysiology of schizophrenia which is characterized with symptomatic onset in early adulthood or late life (Olney et al., 1995). The evidence that glutamatergic dysfunction can help to explain different aspects of schizophrenia are presented below.

2.3.1 Psychotomimetic drugs

It has been shown that various NMDA antagonists, both competitive and non-competitive, can cause psychotic reactions in human as well as neurodegenerative changes in corticolimbic regions of the rat brain (Olney et al., 1995).

Three decades ago, phencyclidine (PCP) was introduced into clinical medicine as an anaesthetic agent but it was soon withdrawn because it caused a high incidence of acute psychotic reactions. Phencyclidine, a non-competitive antagonist of the NMDA subtype of glutamate receptors, produces a schizophreniform psychosis which can consist of both negative and positive symptoms of schizophrenia, such as

catatonia, hebephrenia and schizophrenic thought disorder (Allen et al., 1978; Javitt et al., 1991). It is considered to produce a better drug-induced human model of schizophrenia than that found in amphetamine psychosis which consists primarily of paranoid delusions (Bell, 1965). In addition, researchers in this field were also impressed with the ability of PCP to trigger a prolonged recrudescence of the acute psychotic state in stabilized chronic schizophrenic patients. In the U.S.A. during the past 30 years, PCP has become a not uncommon drug of abuse, but it is rarely encountered in Europe.

Ketamine, a short-acting dissociative anaesthetic agent which blocks the NMDA receptor channel on its phencyclidine-binding site, also produces an acute psychotic reaction. It still gained acceptance as an anaesthetic agent in clinical use because these untoward reactions could be suppressed by benzodiazepines or prevented by barbiturates (Reich, et al., 1989). Although ketamine causes symptoms such as dissociation which is not observed in schizophrenia, it also precipitates positive symptoms, negative symptoms, and cognitive deficits in normal subjects similar to those seen in individuals with schizophrenia.

Three competitive NMDA antagonists ((±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, CPP, CPPene, CGS 19755) that block NMDA receptors by acting at the NMDA or glutamate (rather than PCP) recognition site have now been administrated to human subjects and each was found in low dosage to induce a PCP-like psychotic reaction (Olney et al, 1995). Therefore, the phenomenon known for decades as "PCP psychosis" is not a phencyclidine receptor-specific phenomenon but a phenomenon that results from effective blockade of the NMDA receptor channels.

2.3.2 Glutamate-dopamine interactions in the brain

The hypothesized dysfunction of glutamatergic neurotransmission in schizophrenia can be hypothesized to interdigitate with the traditional "dopamine hypothesis" of schizophrenia. Presynaptic D_2 receptors on corticostriatal and limbic glutamatergic terminals provide a negative regulation of glutamate release (Schwarcz et al., 1978; Kerkerian et al., 1987). Neuroleptic blockade of these presynaptic receptors may thereby enhance excitatory glutamatergic input to the caudate and putamen and to other forebrain regions receiving dopaminergic innervation. In

addition, glutamatergic neurons have reciprocal interactions with dopaminergic processes. The descending pathways of the prefrontal cortex (PFC) modulate the release of dopamine in subcortical areas such as the striatum and this control occurs primarily through glutamatergic projections to the dopamine cell body such as the ventral tegmental area (VTA) rather than the terminal regions such as the dorsal and ventral regions of the medial striatum (Karreman and Moghaddam, 1996). Furthermore, the glutamate-dopamine interactions may occur at the level of the individual neuron (Krebs et al., 1991). These interactions are also consistent with the behavioural studies which showed that the locomotor effects of apomorphine, a dopamine receptor agonist, could be markedly enhanced by co-treatment with the NMDA receptor antagonist MK-801 (Carlsson et al., 1989; 1991) while the MK-801 induced hyperactivity could be attenuated by the dopamine receptor antagonist haloperidol (Dai et al., 1995).

Studies of immediate early gene (IEG) expression provided more evidence for glutamate/dopamine interaction. IEGs are regulatory factors that alter neuronal gene expression as a consequence of neuronal activation such as activation by neurotransmitter receptors. Activation of NMDA receptors is a potent stimulus for turning on IEG expression. Neuroleptics, which are basically D_2 receptor antagonists, induce expression of IEGs in the striatum, nucleus accumbens, and corticolimbic structures and this IEG response to neuroleptics can be reversed by co-administration of NMDA receptor antagonist (Robertson et al., 1992). Systemic treatment of haloperidol-pretreated rats with D-cycloserine, a partial agonist at the glycine modulatory site on the NMDA receptor, augments IEG induction in the striatum (Coyle, 1996). Thus, D-cycloserine behaves like a neuroleptic in terms of its effect on the IEG induction, although it acts on the NMDA receptor rather than the D_2 dopamine receptor.

2.3.3 Implications for the developmental hypothesis of schizophrenia

As mentioned in Chapter one, accumulating evidence suggests that structural changes in cerebrocortical and limbic brain regions are a characteristic of schizophrenia (Weinberger, 1984; Benes et al., 1986; Bogerts 1993; Wolf et al., 1993; Olney et al., 1995) but opinions vary concerning whether such changes occur

during development, during adulthood, or both. If they occur only during early development, why symptoms are not expressed until early adulthood, and if structural changes do not occur in adulthood, how can cognitive deterioration that occurs in some schizophrenics be explained? The potential excitotoxic effect of glutamate during brain development could provide a possible explanation for the anatomical brain abnormalities in schizophrenics such as the increase in volume of cerebral sulci and ventricles as well as a reduction in medial temporal lobe structures (Weinberger, 1984; Bogerts, 1993; Wolf et al., 1993). The glutamatergic dysfunction hypothesis for schizophrenia also resolves these quandaries in that it provides for structural changes of two different types: one that occurs during development and has latent glutamatergic dysfunction potential and another that occurs during adulthood would represent the delayed pathological expression of the earlier structural change (Farber et al., 1995; Olney et al., 1995). Farber et al. (1995) have shown that fetal rats and postnatal rats younger than one and a half months, which is when the first oestrus cycle (menarche) occurs and is roughly equivalent to puberty in the rat (Bennett et al., 1970), are totally insensitive to the cerebrocortical neurotoxic action of NMDA antagonists. Rats between puberty (age 1 1/2 months) and full adulthood (ages 3 and 4 months) gradually become fully sensitive to this toxic mechanism. Similarly, the incidence of acute psychotic reactions associated with ketamine-induced anaesthesia is age-dependent with the reaction occurring rarely in prepubertal children but manifesting in nearly 50% of young and middle-aged adults (Olney et al., 1995). Case reports that have indicated a lack of susceptibility of children to the psychotic effects of PCP suggest a similar age-dependent profile for this entire class of psychotogenic agents (Karp, et al, 1980). These observations seem to fit to the pattern of symptom expression in schizophrenia during late adolescence or early adulthood.

In addition, there are many possible mechanisms by which NMDA receptor-bearing GABAergic neurons might be destroyed in the developing brain, consequently, GABAergic inhibitory control over the activity of corticolimbic neurons is impaired. There is evidence (McDonald et al., 1988) that excitotoxic neuronal degeneration in the developing central nervous system is primarily mediated by NMDA receptors and that different groups of NMDA receptor-bearing neurons

may be hypervulnerable to excitotoxic degeneration at different times in development. This hypothesized mechanism is further supported by the reported evidence documenting selective loss of GABAergic neurons in the cerebral cortex of schizophrenic brains (Benes et al., 1995).

2.3.4 Findings from postmortem schizophrenic brains

In 1980, Kim et al. found that the glutamate concentration in the cerebrospinal fluid (CSF) in twenty schizophrenic patients, among whom 16 were chronic schizophrenics, was about half of the normal value. However, later studies (Perry, et al., 1982; Gattaz, et al., 1985) led to inconsistent results. Postmortem studies of brain glutamate concentrations revealed no change in schizophrenia or a reduced level in only the superior temporal and angular cortices, among 40 areas analyzed (Toru et al., 1994). Because it is technically difficult to measure the exact neurotransmitter pools of glutamate in the brain, these data may not be directly related to the hypothesis. Sherman et al. (1991) found that the release of glutamate was reduced in the synaptosomes from the frontal cortex of schizophrenics. This decrease in glutamate release is calcium ion dependent and supports the glutamate dysfunction hypothesis.

Receptor binding studies in schizophrenic brains report varying abnormalities in glutamate receptor distribution in several brain areas. These results are summarised in Tables 2.1-2.3.

The recent cloning of several glutamate receptor subunits makes it possible to study potential disturbances of glutamate receptors in the schizophrenic brain at the molecular level. Preliminary findings of a regionally specific lowering of the expression of both NMDA and non-NMDA glutamate receptor genes have been reported in the post mortem brain tissue of schizophrenics, for example, an increase in expression of the NMDAR2D subunit mRNA in prefrontal cortex (Akbarian & Jones, 1996), decreased hippocampal expression of mRNAs encoding GluR1 (Harrison et al., 1991) and GluR2 (Harrison et al., 1991; Eastwood et al., 1995), and reductions in the hippocampal immunoreactivity of both GluR2 and GluR3 glutamate receptors (Breese et al., 1995).

Table 2.1 NMDA receptor binding in postmortem schizophrenic brains

Ligand/Displacer	Changes	References
<u>NMDA (Glu) site</u>		
^{3}H-Glu/Glu	No change: 6 areas of bilateral hippocampus	Kerwin et al., 1990
<u>Within the channel</u>		
^{3}H-MK-801/MK-801	Increase: putamen No change: frontal cortex, entorhinal cortex, hippocampus	Kornhuber et al., 1989
^{3}H-MK-801/MK-801	Increase: superior temporal cortex, superior parietal cortex, supramarginal cortex	Suga et al., 1990
^{3}H-TCP/ketamine	Increase: bilateral orbital frontal cortex (BA11) No change: anterior frontal cortex (BA10), temporal cortex (BA38), amygdala	Simpson et al., 1992
<u>Strychnine-insensitive glycine site</u>		
^{3}H-glycine/glycine	Increase: premotor cortex somaesthetic cortex, supramarginal cortex, angular cortex, visual areas 1, 2, 3 No change: prefrontal cortex, motor area, temporal cortex, superior parietal cortex	Ishimaru et al., 1994

Table 2.2 AMPA receptor bindings in postmortem schizophrenic brains

Ligand/Displacer	Changes	References
^{3}H-CNQX/Glu	Decrease: bilateral CA4, left CA3 No change: dentate, right CA1,2,3, parahippocampal gyrus	Kerwin et al., 1990
^{3}H-AMPA/Glu	No change: frontal, temporal, parietal, occipital, limbic cortices	Kurumaji et al., 1992
^{3}H-AMPA/Glu	No change: frontal cortex, cortex, superior parietal cortex, supramarginal cortex	Freed et al., 1993

Table 2.3 Kainate (KA) receptor binding in postmortem schizophrenic brains

Ligand/Displacer	Changes	References
^{3}H-KA/Glu	Increase: med. frontal lobe, angular cortex No change: orbitofrontal area, parietal and occipital cortices	Nishikawa, 1983
^{3}H-KA/Glu	Decrease: left hippocampus No change: right hippocampus	Kerwin et al., 1988
^{3}H-KA/Glu	Increase: orbital cortex No change: prefrontal cortex (BA21,22,38), hippocampus amygdala	Deakin et al., 1989
^{3}H-KA/Glu	Decrease: bilateral dentate gyrus, bilateral CA3,4, parahippocampal gyrus, left CA1,2 No change: right CA 1,2	Kerwin et al., 1990

2.3.5. Alteration of glutamate receptor subunit gene expression in response to neuroleptics

Currently, there are very few studies published on the expression of the NMDA receptor subunit genes following antipsychotic drug treatment. Oretti and Buckland (1994) did not find any significant change of NMDAR1, 2A, 2B, 2C mRNA levels in the whole rat brain after treatment with haloperidol for 32 days. Fitzgerald and Nestler (1995) found that treatment with haloperidol, a more D_2-like antagonist, for 30 days significantly increased NMDAR1 subunit immunoreactivity in the striatum and posterior cingulate cortex while SCH 23390, a potent D_1-like antagonist, decreased the receptor immunoreactivity in the striatum and frontal-parietal cortex. However, Tarazi and Creese (1996) found that subchronic (28 days) treatment with haloperidol or clozapine reduced ^{3}H-MK801 binding in the medial prefrontal cortex; both subchronic and chronic (8 months) treatment with clozapine also reduced ^{3}H-MK801 binding in the caudate putamen; yet subchronic SCH23390 treatment elevated ^{3}H-MK801 binding in the hippocampal formation.

There are even fewer studies on the expression of the AMPA or kainate subtypes of glutamate receptor. Using in situ hybridization technique, Meador-Woodruff et al., (1996) found that mRNA levels of GluR1 in the rat hippocampus were increased after treatment with either haloperidol (2 mg/kg/day) or clozapine (20 mg/kg/day) for 14 days, yet Eastwood et al. (1994) did not find any change under the same conditions. Oretti et al. (1994) measured the mRNA levels by multi-probe oligonucleotide solution hybridization (MOSH) and also found no change. Meador-Woodruff et al., (1996) showed that clozapine (20 mg/kg/day) upregulated the expression of GluR5,6,7 subunits of kainate receptor in the hippocampus. However, Tarazi et al., (1996) did not find any change of AMPA or kainate receptor protein in a binding study.

Despite these contradictory results, these studies appear to demonstrate that glutamate receptor subunits are involved, through direct or indirect mechanisms, in the successful treatment of schizophrenia.

2.3.6 Clinical trials of glycine/D-cycloserine therapy

There have been several clinical reports on the efficacy of glycine in the

amelioration of symptoms in some schizophrenics previously unresponsive to neuroleptics or of negative symptoms of this disease (Waziri, 1988; Javitt et al., 1994; Leiderman et al., 1996; Heresco-Levy et al., 1996). These observations are of particular interest because glycine therapy may involve its role as a co-agonist with glutamate at the strychnine-insensitive glycine site of the NMDA receptor.

Although effects of glycine could also be due to its strychnine-sensitive inhibitory effects on dopaminergic neurons in the substantia nigra (Cheramy et al., 1978; Waziri, 1996), other clinical trials of D-cycloserine (Goff et al., 1995; 1996) have provided further evidence of its action through the glycine site of the NMDA subtype of the glutamate receptor. D-cycloserine is a partial agonist at the glycine site of the NMDA receptor that exhibits agonist properties over a narrow dose range and antagonizes the effects of glycine. Since D-cycloserine readily crosses the blood-brain barrier, it provides a useful probe for testing the hypothesis of NMDA hypofunction in schizophrenia. The results from a dose-finding trial (Goff et al., 1995) of D-cycloserine added to conventional neuroleptics in which a daily dose of 50 mg of D-cycloserine significantly improved negative symptoms are consistent with evidence that D-cycloserine only displays agonist properties over a narrow dose range (Emmett et al., 1991) and high dose (250 mg/day) of D-cycloserine did not produce therapeutic effects when added to conventional neuroleptics.

Compared with the results from the clinical trial of D-cycloserine with conventional or so-called "typical" neuroleptics, the effect on negative symptoms and cognitive function of adding D-cycloserine to clozapine is different. The improvement of negative symptoms and cognitive function with 50 mg/day of D-cycloserine in patients treated with conventional neuroleptics may not occur in patients treated with clozapine (Goff et al., 1996). Clozapine has been thought to differ from conventional neuroleptics in its effects on NMDA receptors. For example, social isolation produced in rats by the administration of the NMDA antagonist phencyclidine was attenuated by clozapine but not by conventional neuroleptics (Corbett, et al., 1995). The basal serum concentrations of glutamate in clozapine-treated patients were significantly higher than those in conventional neuroleptic treated patients, yet the glycine levels in the clozapine-treated patients were lower before they began taking D-cycloserine (Goff, et al., 1996). These

findings suggest that D-cycloserine would not produce additional augmentation of the effect of clozapine in improving negative symptoms because the NMDA receptor-mediated systems are already activated by clozapine.

Although large-scale application of glycine therapy in schizophrenia can not be clinically desirable before the possible neurotoxic effects of glycine is ruled out, these clinical studies have provided some evidence of the involvement of the NMDA subtype of glutamate receptor in schizophrenia, especially in the production of negative symptoms and cognitive impairment of this illness.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials and methods in genetic linkage studies

3.1.1 Family sampling and diagnostic procedures

Twenty-three pedigrees (11 Icelandic and 12 English) containing cases of schizophrenia were studied. Diagnoses were assigned according to the Research Diagnostic Criteria, RDC (Spitzer, 1978a). Subjects were interviewed with the Schedule for Affective Disorders and Schizophrenia--the Lifetime Version, SADS-L (Spitzer, 1978b). Information from this interview was supplemented by material from casenotes. Extensive tracing of pedigrees was carried out and attempts were made to characterize as far as possible the diagnoses of other members of the kindreds. Subjects were also rated for schizoid personality and schizotypal disorder according to the DSM-IIIR criteria. Pedigrees were included on the basis of containing multiple cases of schizophrenia but no cases of bipolar affective illness and of appearing to demonstrate unilineal inheritance. Two psychiatrists who were blind to genotyping assigned consensus diagnoses.

Two affection classes were used for the linkage analysis: 1) "core schizophrenia" consists of schizophrenia, unspecified functional psychosis and schizoaffective psychosis, and 2) "schizophrenia spectrum" consisting additionally of schizoid and schizotypal personality disorder according to DSM-IIIR criteria. Of the 375 interviewed individuals in these 23 pedigrees, 95 fell into the core schizophrenia category, an additional 18 fell into the schizophrenia spectrum category.

3.1.2 Genotyping

Genomic DNA was extracted from blood samples obtained from 271 available members of these 23 families. 50 ng of total genomic DNA was amplified by polymerase chain reaction (PCR) with oligonucleotide primers for GluR5, GluR6 glutamate receptor subunit genes, and SLC1A5 which is the gene encoding a neutral amino acid glutamate/aspartate transporter respectively. One primer of each set was 5' end labelled using T_4 polynucleotide kinase (Boehringer Mannheim) with τ-^{32}P-

ATP (Amersham). The PCR solution was in a total volume of 12.5 ul containing 1.0 picomole of each primer (Oswell), 200 uM each of dATP, dTTP, dCTP, dGTP (Pharmacia), 1.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% w/v gelatin and 0.5 unit Taq polymerase (Boehringer Mannheim). Details of the amplification conditions and characteristics of each primers were summarized in Table 3.1. The alleles were separated by electrophoresis in 6% denaturing polyacrylamide DNA sequencing gels. The gels were fixed, dried then exposed to high sensitivity X-ray films (Fuji, Japan) at -70°C.

Genotypes were read by two independent assessors blind to diagnostic data, who came to a consensus over discrepant genotypes.

Table 3.1 PCR conditions and sequence of the oligonucleotide primers used for GluR5, GluR6, and SLC1A5

	Primer sequence	PCR conditions	PCR Products	Chromosomal localization	Reference
GluR5	5'GCTAAATAGATATATGATAAACGG3'	94°C 1 min.	AGAT tetranucleotide	21q22.1	Gregor (1994)
	5'CTGGCAGTAAATGTCTATGAAAC3'	56°C 1 min.	repeat		
		72°C 1 min.	6 alleles		
		32 cycles			
GluR6	5'CAACACCTTTTCTCTAACCCC3'	94°C 1 min.	TAA trinucleotide repeat	6	Paschen (1994)
	5'CTCGGCCAGTTTTTACAACTTG3'	65°C 50 sec.	7 alleles	6q21-22*	Gregor (1993)
		72°C 1 min.		*predicted from mouse genetics	
		25 cycles			
SLC1A5	5'CTGTTATTGTGGAGGGAATAG3'	94°C 1 min.	GT dinucleotide repeat	19q13.3	Jones (1994)
	5'GGGATGTTACAACACCATGC3'	58°C 1 min.	7 alleles		
		72°C 1 min.			
		25 cycles			

3.1.3 Linkage analysis

Linkage analyses were carried out with the use of FASTLINK (Lathrop et al., 1985; Cottingham et al., 1993), ERPA (Curtis and Sham, 1994) and MFLINK (Curtis and Sham, 1995).

3.1.3.1 Lod score analysis (FASTLINK)

To carry out lod score analyses, for the core schizophrenia model the penetrance for individuals carrying the disease allele was set at 0.73, while allowance for sporadic cases was made by setting the penetrance for normal homozygotes at 0.005. For the schizophrenia spectrum model these penetrance values were set at 0.76 and 0.01 respectively. Analyses were carried out assuming dominant transmission with the gene frequency of the disease allele set at 0.0085.

3.1.3.2 Lod2 statistic

To investigate the possibility that only a subset of pedigrees might have a susceptibility locus in the region studied, the lod2 statistic was used (Risch, 1989). This is the log of the ratio of the likelihoods under the assumption that a proportion of families are linked at a certain recombination fraction against the likelihood under the assumption that the recombination fraction is 0.5 between disease and marker in all families. This lod2 statistic is thus similar to a lod score but includes an extra degree of freedom for alpha, the proportion of families which are linked.

3.1.3.3 Extended relative pair analysis, ERPA

To test for linkage without making any assumptions about mode of transmission, ERPA and MFLINK analyses were performed. ERPA (extended relative pair analysis) is a nonparametric method which is based on identity-by-descent (IBD) relationships between all pairs of affected relatives within a pedigree (Curtis and Sham, 1994). This approach is an extension of extended sib pair analysis (ESPA, Sandkuijl, 1989) which only uses information from pairs of affected siblings. The ERPA method uses the risk calculation facilities of the LINKAGE programs to obtain the necessary information in a fashion which is simple to implement and which is automatically generalizes to allow for marker loci which

may be multiple, non-codominant and sex-linked.

3.1.3.4 Model-free linkage analysis, MFLINK

MFLINK (Curtis and Sham, 1995) does not rely on specification of a particular mode of transmission. It compares the likelihoods for the observed data under the hypothesis that a locus at a particular test position influences susceptibility in a proportion of families (i.e. linkage) and under the hypothesis that it has no effect (i.e. non-linkage). Both likelihoods are maximised independently over a wide range of transmission models, ranging from Mendelian dominant to null effect and from null effect to Mendelian recessive, and the likelihood under linkage is additionally maximised over alpha, the proportion of families linked. The difference between maximised log-likelihoods forms a "model-free" lod score. One position was tested, at a recombination fraction of 0.01 with the each gene to be tested. MFLINK also reports the maximum lod scores obtained for any transmission model under the assumptions of homogeneity or admixture.

3.2 Materials and methods in gene expression studies

3.2.1 Pharmacological characteristics of flupenthixol

Flupenthixol is an antipsychotic drug of the thioxanthene group which exhibits geometric isomerism. There is a stereospecific interaction with certain neuroreceptors and a correlation with its clinical potency. The cis-isomer of flupenthixol has a 50-fold greater potency in blocking the dopamine receptor than its trans-isoform (Enna et al., 1976). Clinical studies also indicated a significant superiority of the cis isomer over the trans form in its ability to ameliorate the positive symptoms of schizophrenia (Johnstone et al., 1978). This approach permits a clinically relevant analysis of the regulatory characteristics of individual glutamate receptor subunits at the level of gene expression and should lead to a better understanding of the molecular actions of antipsychotic drugs as well as the possible pathophysiology of schizophrenia. Pharmacological characteristics of trans- and cis-

flupenthixol in several neurotransmitter receptor systems were summarized in Table 3.2.

Table 3.2 Relative potencies of cis- and trans- flupenthixol in several neurotransmitter receptor systems

	Cis- (α, Z)	**Trans- (ß, E)**
Dopamine (DA) receptors:		
Dopamine binding (Enna & Snyder, 1976)	50	1
Haloperidol binding (Enna & Snyder, 1976)	50	1
DA Adenylate cyclase (Miller, 1974)	1000	1
SCH-23390 (D_1) binding	29	1
Spiperone (D_2) binding	20	1
D_1 : D_2 potency ratio (Faedda & Baldessarini, 1989)	1.58	1.08
Noradrenaline (NA) receptors:		
NA Adenylate cyclase (Miller, 1974)	1	1
WB-4101 (NA α) binding (Peroutka & Snyder, 1977)	3.6	1
Serotonin (5-HT) receptors:		
Serotonin binding	15	1
LSD binding (Enna & Snyder, 1976)	60	1

(Table 3.2, continued)

Muscarinic receptors:		
QNB binding	1	1
(Enna & Snyder, 1976)		
Opiate receptors:		
Naloxone binding	1	1
Dihydromorphine binding	1	1
(Enna & Snyder, 1976)		
GABA receptors:		
Inhibition of GABA uptake	1	1
(Enna & Snyder, 1976)		

3.2.2 Chronic drug treatments

Adult male Sprague-Dawley rats (initial weight 225-250 grams, Biological Services, University College London) were housed 3 per cage under a 12 hr. light-dark cycle and permitted food and water *ad libitum*. A total of 144 rats were divided into three groups which received chronic treatments with the cis- or trans-isomer of flupenthixol (Lundbeck, Denmark) or no drug as control group. Oral treatment regimens were selected in order to provide more stable and continuous levels of the antipsychotic drugs to mimic the clinical situation. The drug concentrations used were 0.2 mg/kg/day for both cis- and trans-flupenthixol. This dose is equivalent to the recommended clinical dosage by the suppliers and it is also the same dose used in our prevous study (De La Concha et al., 1991) in which a two to three fold increase in the abundance of dopamine D2 receptor mRNA in mouse half brain was observed after treatment with the cis- but not the trans-drug for ten weeks. The drug was delivered in the drinking water for up to 24 weeks in this study. The amount of average water consumption was calculated for several weeks prior to the experiment and it was found to be similar to that described previously (See and Ellison, 1990). The average body weight and water consumption were also

monitored throughout the 24 week period to ensure a stable average oral dosage. Eight rats of each drug-treated or control group were killed at the end of 1, 2, 4, 8, 12, 24 weeks.

3.2.3 Tissue and RNA preparation

Brains were removed rapidly from decapitated rats and the left brain was immediately separated into frontal lobe, parietal cortex, subcortical, and cerebellar regions by gross dissection on ice. The separate brain tissues from the four regions were stored at -70°C until use. Total RNA was isolated and stored according to the new Chomczynski method (Chomczynski, 1993) with an RNA isolator kit (Genosys, USA).

3.2.4 Multi-probe oligonucleotide solution hybridization (MOSH)

Each rat brain RNA sample from the four left brain regions was analysed individually using multi-probe oligonucleotide solution hybridization (MOSH) as described previously (Buckland et al., 1992). Briefly, oligonucleotide probes were end labelled with r ^{32}P-ATP by T4 polynucleotide kinase with the KinaseMax 5'end-labelling kit (Ambion, USA) then a gel purification was further performed. The sequences of the probes used for ß-actin, NMDAR1, 2A, 2B, 2C, and GluR1, 2, 3,4 receptor subunit genes were according to those employed by Oretti et al. (1994). The sequence of the probe for the NMDAR2D is as follow: 5'- GGC AGG TAG TCC CAG CTA CCA GCG CGC -3'. This probe is complementary to nucleotides 3,483-3,509 of the NMDAR2D-1 variant (EMBL/Genbank accession no. D13213) or to nucleotides 4,189 to 4,215 of the NMDAR2D-2 variant (EMBL/Genbank accession no. L31612), i.e., this probe can detect both NR2D splice variants either with (NR2D-1) or without (NR2D-2) a deletion which is possibly due to alternative splicing and leads to different amino acid sequences in their C-terminal (Ishii et al., 1993). The sequences of the probes used for GluR7, KA1 and KA2 glutamate receptor subunit genes were modified from the probes previously used for in situ hybridization (Bettler and Heinemann, 1992; Werner and Seeburg, 1991; Herb and Seeburg, 1992). Details of the probes used were summarised in Table 3.3.

0.1 pmole of each probe and 20 ug of total RNA were suspended in 30 ul

hybridization buffer with 0.4 M NaCl, 40 mM disodium salt of PIPES (1,4-piperazinediethanesufonic acid) pH 6.4, 1 mM EDTA adjusted pH with NaOH to pH 6.4, denatured at 90°C for 3 minutes and incubated at 65°C for 2 hours. Excess unhybridized probes were then digested and removed by the addition of 300 ul S1 nuclease digesting buffer (zinc sulphate 4.5 mM, sodium acetate 50 mM pH 4.2, NaCl 0.3 M, and 10 ug/ml single-stranded DNA as carrier DNA) with the S1 enzyme concentration of 100 U/ml at 37°C for 15 minutes. Double stranded probe:RNA hybrids were precipitated with ethanol, resuspended in 10 ul formamide running buffer, denatured at 90°C for 2 min, subjected to denaturing polyacylamide gel electrophoresis and then visualized on X-ray film autoradiography (Fuji, Japan).

However, like most other RNA protection assays, it can be difficult to set up optimal conditions for MOSH. A few points had to be addressed before this technique was finally working in the Molecular Psychiatry Laboratory: 1) The quality of RNA is very important. As mentioned in the previous paragraph, partially degraded RNAs would be fine for the use of MOSH. However, the resulting RNA should be DNA- and guanidine salt-free. It is therefore recommended to precipitate RNAs with ethanol at room temperature rather than at -20°C as many RNA preparation protocols suggest because the lower temperature usually causes co-precipitation of salts with RNAs. 2) It is generally acknowledged that a DNA:RNA duplex is not as stable as an RNA:RNA duplex. Therefore, end-labelled oligonucleotide probes were further gel-purified before use. In addition, the hybridization temperature (65°C) was generally higher than those described in the literature in order to reduce the chance of non-specific hybridization occurring. 3) Titration of probe/ RNA/ S1 enzyme concentrations needs to be performed before experiments are carried out. S1 nuclease is an aggressive enzyme in that it can degrade double-stranded DNA:RNA duplexes as well if the enzyme is in excess. On the other hand, if the enzyme concentration is too low, it can result in an incomplete digestion of the single-stranded unhybridized probes which hamper the accuracy of measurement of the target mRNA levels. We used 20 ug of total RNA per tube with 100 U/ml of S1 nuclease. Some other groups (Buckland et al., personal communication) have tried the same conditions or have used 10 ug of total RNA with 80 U/ml of S1 enzyme and both conditions were found work. Figure 3.1

shows a titration result.

In addition to the 'blank experiment' carried out with every MOSH experiment as a "negative control" (Figure 5.1), the results obtained also reflect the relative abundance of each target mRNA. For example, generally the mRNA for ß-actin was the most abundant amongst those genes being tested in this study. The order of abundance was NR1, NR2B/NR2A, NR2C/NR2D from observations in most brain regions of the control rats. NR2C mRNA was expressed in a relative low amount in most brain regions but its expression was high in cerebellum (Figure not shown). These abundance differences of mRNA expression either between different target genes in the same brain region or between the same target gene in different brain regions are consistent with other results reported previously (Monyer et al., 1992; Ishii et al, 1993). The upregulation of D_2 receptor mRNA by flupenthixol found in these results (Table 5.13) could also be served as a "positive control" for the MOSH experiment. Overall, these results confirmed the considerable consistency and reliability of the newly-developed MOSH method for determination of the relative abundance of specific mRNAs.

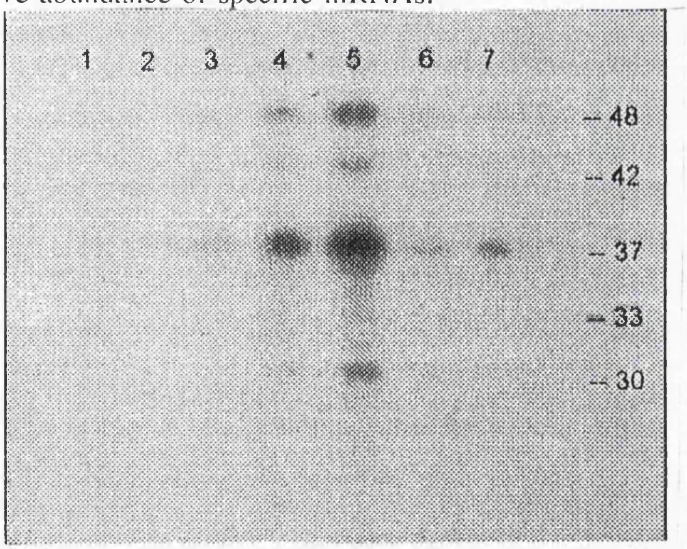

Fig 3.1 A titration result from MOSH. 100 U/ml of S1 enzyme was added for each experiment. The size of each probe used is as follows: NMDAR1: 48 mer, NMDAR2A: 42 mer, ß-actin: 37 mer, NMDAR2C: 33 mer, NMDAR2B: 30 mer. Lane 1: no RNA. Lane 2: 40 ug of t-RNA. Lane 3: 10 ug of rat brain RNA. Lane 4: 20 ug of rat brain RNA. Lane 5: 40 ug of rat brain RNA. Lanes 6 and 7 were loaded with "poorly-prepared rat brain RNA" (20 and 40 ug respectively) which was precipitated at -20°C instead of room temperature hence contaminated with guanidine salts. It can be seen that Lanes 3-5 show a good linear correlation between signals and total RNA concentrations.

Table 3.3 Sequence of oligonucleotide probes used in MOSH

Target gene	Sequence	Size	Reference
ß-actin	5'- CGT TGT CGA CGA CGA GCG CAG CGA TAT CGT CAT CCA T -3'	37 mer	Oretti 1994
NMDAR1	5'- GCC TCG CGG AAC ATC TGT TCA TGC TTG CGC GTG CTC AGC ACC GCG CCG -3'	48 mer	Oretti 1994
NMDAR2A	5'- TAT CCC AGC CGG AGG CTC TGC AGC AGG GCT CGC AGC CTC TCT -3'	42 mer	Oretti 1994
NMDAR2B	5'- CGC TGG GCT TCA TCT TCA GCT AGT CGG CTC -3'	30 mer	Oretti 1994
NMDAR2C	5'- CTG CCA AAC ACC ACC GCC ACA GTC ACG GCC TGT -3'	33 mer	Oretti 1994
NMDAR2D	5'- GGC AGG TAG TCC CAG CTA CCA GCG CGC -3'	27 mer	Ishii 1993
DRD2	5'- GTC ATC GTA CCA GGA CAG GTT CAG TGG -3'	27 mer	Buckland 1993a,b
GluR1	5'- CCC ACA ACC GCA CCT AGA AAA CCG GTG CAG -3'	30 mer	Oretti 1994
GluR2	5'- CAG CAG GTC CCC ATC AGT GAA TCC CAG ATT TGC -3'	33 mer	Oretti 1994
GluR3	5'- TCC ACC TAT GCT GAT GGT GTT GGG GAA TCC TCC GTG AGA ATG -3'	42 mer	Oretti 1994
GluR4	5'- GAA GAG ACC ACC TAT TTG AAC GCT GCT TGG AAA GGC TCC CAT GGC GAG -3'	48 mer	Oretti 1994
GluR5	5'- CGA CTG GGG TTA TGG ATA AAG ACA TAG -3'	27 mer	Bettler 1990
GluR6	5'- GCT TCA GTC TTC CCT GTA ACC AGT AAC ACT -3'	30 mer	Egebjerg 1991
GluR7	5'- CGA GGA TGG CGT GGC TGA GAG AGG CGT AGT CGG -3'	33 mer	Bettler 1992
KA1	5'- CTT GTA GTT GAA CCG TAG GAT CTC AGC CAA CTC CTT GAG CAT -3'	42 mer	Werner 1991
KA2	5'- GTT CTC CAG GAT ATG GGG ACG CGC CCG AAG ACA CGG GTG AGG GTT ATA -3'	48 mer	Herb 1992

3.2.5 Quantification of mRNA and statistical analysis

All bands visualized on the autoradiographs were analysed using the Molecular Analyst/GS-670 Densitometer (Bio-Rad, USA). The ratio of band intensities of each probe relative to the ß-actin probe were calculated for each sample. ß-actin is a structural gene which is commonly used as an internal standard for gene expression studies. Its expression is not affected by any of the drugs used (De La Concha et al, 1991). To ensure an analysis in the linear range, X-ray films were exposed to a prior MOSH experiment of increasing total RNA concentrations (10, 20, 40 ug per lane) for various times. The resulting signals from X-ray film showed a well linear correlation. The MOSH experiments were carried out for each brain region with simultaneous hybridizations and gel eletrophoresis of mRNA from 8 control, trans- and cis- drug treated rats to be compared. This design permitted a statistical approach to detect gene expression changes thus possible variability in mRNA quantification due to the normal decay of radioactivity and experimental variation of individual autoradiograph should be overcome. The densitometry analyses were carried out by two assessors independently on a subgroup of MOSH samples in order to check the reliability of the most statistically significant gene expression changes. Comparison of the two readings showed a high correlation with no difference in statistical interpretation resulting from independent measurement of expression changes. The normalized intensities of each probe compared to ß-actin from trans- or cis-flupenthixol treated rats were compared with those from corresponding control rats by Wilcoxon's rank sum test using Easistat software (ARC Scientific Limited, UK).

3.2.6 Western (immuno) blot analysis

3.2.6.1 Brain membrane protein preparation

The right rat brains were also immediately separated into frontal lobe, parietal cortex, subcortical, and cerebellar regions by gross dissection on ice as described above. The method for brain membrane processing was reported previously (Jones, et al, 1989). Briefly, right brain tissue samples from the four above-mentioned regions were homogenized respectively in ice-cold 0.32M sucrose with 20 volumes of original wet weight of tissue for 30 seconds with a Polytron. The homogenate

was centrifuged at 1,000 g for 10 minutes at 4°C. The supernatant was centrifuged at 20,000 g for 20 minutes at 4°C. The pellet was resuspended in 20 volumes of ice-cold HPLC grade water and spun at 7,600 g for 20 minutes. The pellet with its upper buffy layer was collected and further washed twice with HPLC grade water by centrifugation at 48,000 g for 20 minutes. The pellet was collected and frozen at -20°C for one to seven days. The resulting pellet was resuspended in 20 volumes ice-cold 50mM HEPES buffer (pH 7.4). The homogenate was preincubated at 37°C for 20 minutes then centrifuged at 48,000 g for 10 minutes at 4°C. The final pellet was resuspended in 50 mM HEPES buffer and protein concentrations were determined by the method of Bradford (Bradford, 1976).

3.2.6.2 Sources and characteristics of the antibodies

All the primary antibodies against each specific glutamate receptor subunit were purchased from Upstate Biotechnology Inc., USA. They are all polyclonal antibodies which were raised against HPLC-purified synthetic peptides corresponding to each receptor subunit respectively. The molecular weights of each glutamate receptor subunits and the amino acid sequences of the corresponding synthetic peptides are as follows: NMDAR1 subunit (M_r 116 kDa), synthetic C-terminal peptide (KRRAIEREEGQLQLCSRHRES) of rat NMDAR1; The anti-rat GluR2/3 antibody recognizes GluR2/3 subunits (M_r 100-105 kDa) from human, rat, mouse, monkey, chicken and the immunogen is a 21-residue synthetic peptide (KQNFATYKEGYNVYGIESVKI) corresponding to the C-terminal of rat GluR2 with a lysine added at the N-terminus; The anti-rat GluR6/7 antibody recognizes the 115 kDa GluR6/7 subunits from human, rat, mouse, monkey, chicken, rabbit and bovine and the immunogen is a 16-residue synthetic peptide (KHTFNDRRLPGKETMA) corresponding to the C-terminal of rat GluR6 with a lysine added at the N-terminus of the peptide.

3.2.6.3 Western blot analysis

Samples (50 ug protein) were subjected to SDS-polyacrylamide gel electrophoresis with 7.5% acrylamide/0.3% bis-acylamide in the resolving gels. Proteins were transferred eletrophoretically to nitrocellulose filters (Hybond-C extra,

Amersham). The blotted nitrocellulose filters were first temporarily stained with Ponceau-S solution (Sigma) to ensure that the protein of each lane was evenly loaded and completely transferred to the nitrocellulose membrane. After washing with water, the filter was then blocked with freshly prepared phosphate-buffer saline (PBS) containing 5% nonfat dry milk (MLK) for 20 minutes at 20-25°C with constant agitation and then was incubated with 1 ug/ml of respective primary antibody in freshly prepared PBS-MLK overnight with agitation at 4°C. After washing twice with PBS-MLK, the nitrocellulose filters were incubated with ^{125}I-labelled Protein A (Amersham) with a final concentration of 0.2 uCi/ml in PBS-MLK for 1.5 hours followed by two washes with PBS and one with PBS-0.05% Tween 20 (Sigma, USA). The filters were then exposed to a phosphoimager screen or X-ray film for 2 days and levels of immunoreactivity were quantitated using a computer-assisted image analyser (the Molecular Analyst, Bio-Rad, USA). To ensure an analysis in the linear range, X-ray films were exposed to western blots of increasing protein concentrations (25, 50, 75, 100, 125, 150 ug per lane) for various times. The resulting signals from X-ray film showed a linear correlation over at least a 3-fold range of tissue concentration. The intensities of western blots from trans- or cis-flupenthixol treated rats were compared with those from corresponding control rats by Wilcoxon's rank sum test using Easistat software (ARC Scientific Limited, UK).

CHAPTER 4: GENETIC LINKAGE STUDIES ON THE GLUTAMATE RECEPTOR SUBUNIT GENES AND SCHIZOPHRENIA

4.1 Results of the linkage studies on the GluR5 glutamate receptor subunit gene

The two-point lod scores obtained with each affection model (core schizophrenia and schizophrenia spectrum) against the GluR5 marker are shown in Table 4.1. It can be seen that the results are very similar for each affection model and that under the assumption of homogeneity the total lod scores are quite negative and result in an exclusion (lod score less than -2) up to a recombination fraction of 20%. In order to investigate the possibility that only a subset of pedigrees might have a susceptibility locus in the region studied, the lod2 statistic was used (Risch, 1989). The lod2 statistic for heterogeneity did not rise above zero for any value of alpha or theta. The results of the ERPA analysis are shown in Table 4.4, and these are negative. The results from MFLINK are also negative with a model-free lod score of 0, and an admixture lod score maximised over transmission models of only 0.1. The overall results obtained exclude the hypothesis that the GluR5 allelic variants provide a major gene contribution to the aetiology of schizophrenia.

4.2 Results of the linkage studies on the GluR6 glutamate receptor subunit gene

The two-point lod scores obtained under the hypotheses of homogeneity and admixture with each affection model (core schizophrenia and schizophrenia spectrum) against the GluR6 gene marker are shown in Table 4.2. It can be seen that the results are very similar for each affection model and that the total lod scores are strongly negative at small recombination fractions and result in an exclusion (lod score less than -2) up to a recombination fraction of 5% under the assumption of homogeneity. The total lod score becomes slightly positive at larger recombination

fractions, and under admixture the schizophrenia spectrum model produces a lod2 of 0.51 at GluR6 assuming 30% of families are linked. 6 of the 23 pedigrees revealed slightly positive individual lod scores at GluR6, with the largest lod scores in any single family being 0.83 for the core schizophrenia model and 0.77 for the schizophrenia spectrum model. The results of the ERPA analyses are shown in Table 4.4, and these show a slight, non-significant excess of allele sharing between affected relatives. MFLINK analysis yielded a model-free lod score of 0.45, and an admixture lod score maximised over transmission models of 0.52.

4.3 Results of the linkage studies on the SLC1A5 glutamate transporter gene

The two-point lod scores obtained with each affection model (core schizophrenia and schizophrenia spectrum) against the SCL1A5 gene are shown in Table 4.3. The results are also very similar for each affection model and that under the assumption of homogeneity the total lod scores are quite negative and result in an exclusion (lod score less than -2) up to a recombination fraction of 20%. The lod2 statistic did not rise above zero for any value of alpha or theta. The results from MFLINK are also negative with a model-free lod score of 0.00 for either the core schizophrenia or the schizophrenia spectrum models, and an admixture lod score maximised over transmission models of only 0.00 for the core schizophrenia model and 0.23 for the schizophrenia spectrum model. These results do not support the hypothesis that the SLC1A5 allelic variants contribute to the aetiology of schizophrenia.

Table 4.1a Two-point lod scores between disease and marker for the **GluR5** locus at specific values of the recombination fraction (theta) using the **core schizophrenia** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-1.941	-1.719	-1.145	-0.746	-0.325	-0.123	-0.028
27	-0.843	-0.844	-0.838	-0.797	-0.558	-0.259	-0.064
35	-2.129	-1.794	-1.215	-0.844	-0.406	-0.163	-0.038
36	-2.488	-2.097	-1.385	-0.936	-0.443	-0.180	-0.043
40	-0.409	-0.379	-0.279	-0.187	-0.076	-0.024	-0.005
41	-1.993	-1.587	-0.727	-0.273	0.077	0.131	0.057
45	-0.547	-0.521	-0.421	-0.310	-0.142	-0.040	0.006
46	0.183	0.177	0.154	0.126	0.075	0.035	0.009
47	0.667	0.652	0.591	0.513	0.349	0.187	0.054
48	0.486	0.474	0.427	0.365	0.241	0.124	0.034
74	-1.974	-1.781	-1.089	-0.644	-0.238	-0.073	-0.013
84	-0.598	-0.556	-0.419	-0.295	-0.138	-0.053	-0.011
121	-2.565	-2.331	-1.805	-1.404	-0.862	-0.486	-0.208
125	-0.563	-0.468	-0.241	-0.100	0.010	0.026	0.010
141	0.077	0.074	0.062	0.048	0.027	0.012	0.003
143	-0.371	-0.346	-0.261	-0.183	-0.083	-0.031	-0.006
152	0.001	0.001	0.001	0.001	0.001	0.001	0.001
153	-0.029	-0.028	-0.025	-0.021	-0.013	-0.006	-0.002
157	-0.318	-0.304	-0.251	-0.194	-0.105	-0.045	-0.011
158	-1.238	-1.056	-0.676	-0.440	-0.195	-0.075	-0.017
184	-1.648	-1.362	-0.843	-0.526	-0.202	-0.063	-0.019
224	-0.026	-0.025	-0.021	-0.017	-0.009	-0.004	-0.001
250	-0.200	-0.194	-0.166	-0.133	-0.076	-0.034	-0.009
total	-18.464	-16.011	-10.573	-6.996	-3.091	-1.142	-0.299
alpha	0.000	0.000	0.000	0.000	0.000	0.000	0.000
lod2	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 4.1b Two-point lod scores between disease and marker for the **GluR5** locus at specific values of the recombination fraction (theta) using the **schizophrenia spectrum** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-1.800	-1.588	-1.078	-0.717	-0.320	-0.122	-0.027
27	-2.748	-2.508	-1.753	-1.125	-0.412	-0.105	-0.014
35	-2.077	-1.829	-1.294	-0.907	-0.437	-0.174	-0.041
36	-1.676	-1.393	-0.816	-0.467	-0.146	-0.031	-0.002
40	-0.499	-0.463	-0.343	-0.233	-0.099	-0.034	-0.007
41	-3.097	-2.489	-1.389	-0.800	-0.295	-0.103	-0.024
45	-0.287	-0.278	-0.241	-0.192	-0.099	-0.033	0.001
46	-0.985	-0.857	-0.562	-0.370	-0.169	-0.067	-0.016
47	0.678	0.663	0.601	0.522	0.356	0.191	0.056
48	0.490	0.478	0.430	0.368	0.243	0.125	0.035
74	-1.913	-1.678	-1.028	-0.612	-0.224	-0.067	-0.011
84	-0.339	-0.319	-0.248	-0.179	-0.087	-0.034	-0.007
121	-2.336	-2.154	-1.711	-1.357	-0.852	-0.486	-0.210
125	-0.409	-0.355	-0.204	-0.096	-0.002	0.016	0.007
141	0.215	0.207	0.178	0.143	0.083	0.038	0.010
143	-1.395	-1.230	-0.818	-0.526	-0.214	-0.068	-0.009
152	0.085	0.082	0.071	0.057	0.034	0.016	0.004
153	0.017	0.017	0.018	0.017	0.012	0.006	0.002
157	-0.181	-0.175	-0.149	-0.119	-0.067	-0.030	-0.008
158	-0.934	-0.841	-0.588	-0.398	-0.183	-0.071	-0.016
184	-1.312	-1.104	-0.669	-0.396	-0.135	-0.052	-0.045
224	-0.213	-0.202	-0.160	-0.112	-0.045	-0.013	-0.002
250	-0.092	-0.090	-0.079	-0.065	-0.039	-0.018	-0.005
total	-20.809	-18.106	-11.833	-7.562	-3.095	-1.113	-0.328
alpha	0.000	0.000	0.000	0.000	0.000	0.000	0.000
lod2	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 4.2a Two-point lod scores between disease and marker for the **GluR6** locus at specific values of the recombination fraction (theta) using the **core schizophrenia** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-1.204	-0.901	-0.434	-0.194	0.013	0.080	0.069
27	-1.506	-1.093	-0.447	-0.118	0.108	0.103	0.031
35	-0.213	-0.208	-0.187	-0.157	-0.096	-0.045	-0.011
36	-0.001	-0.001	-0.001	-0.001	-0.001	-0.000	-0.000
40	-2.679	-2.318	-1.448	-0.937	-0.426	-0.169	-0.040
41	-2.606	-1.541	-0.827	-0.498	-0.200	-0.088	-0.044
45	-0.415	-0.383	-0.275	-0.180	-0.067	-0.016	-0.001
46	-0.031	-0.029	-0.025	-0.019	-0.011	-0.005	-0.001
47	-0.082	-0.079	-0.070	-0.058	-0.035	-0.016	-0.004
48	0.486	0.474	0.427	0.365	0.241	0.124	0.034
74	0.479	0.466	0.416	0.352	0.227	0.114	0.031
84	-0.273	-0.260	-0.211	-0.161	-0.085	-0.037	-0.009
121	-0.008	-0.008	-0.007	-0.007	-0.005	-0.003	-0.001
125	0.834	0.812	0.723	0.609	0.380	0.175	0.040
141	0.060	0.058	0.049	0.039	0.023	0.010	0.003
143	-0.450	-0.430	-0.355	-0.273	-0.147	-0.063	-0.016
152	-0.008	-0.007	-0.006	-0.005	-0.003	-0.002	-0.001
153	-0.013	-0.013	-0.011	-0.010	-0.006	-0.003	-0.001
157	-0.002	-0.002	-0.002	-0.001	-0.001	-0.000	-0.000
158	0.591	0.576	0.512	0.431	0.273	0.132	0.031
184	0.443	0.437	0.409	0.368	0.266	0.149	0.044
224	-0.008	-0.008	-0.006	-0.005	-0.003	-0.001	-0.000
250	-0.006	-0.005	-0.005	-0.004	-0.003	-0.001	-0.000
total	-6.611	-4.463	-1.784	-0.463	0.445	0.437	0.155
alpha	0.250	0.250	0.300	0.400	0.800	1.000	1.000
lod2	0.389	0.380	0.366	0.386	0.478	0.437	0.155

Table 4.2b Two-point lod scores between disease and marker for the **GluR6** locus at specific values of the recombination fraction (theta) using the **schizophrenia spectrum** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-1.007	-0.798	-0.402	-0.178	0.024	0.088	0.074
27	-1.290	-1.030	-0.528	-0.242	-0.016	0.025	0.008
35	-0.232	-0.227	-0.205	-0.173	-0.107	-0.050	-0.013
36	-0.001	-0.001	-0.001	-0.000	-0.000	-0.000	-0.000
40	-2.517	-2.176	-1.401	-0.920	-0.422	-0.169	-0.040
41	-1.769	-1.651	-1.166	-0.743	-0.283	-0.091	-0.031
45	-0.164	-0.154	-0.119	-0.081	-0.030	-0.005	0.001
46	0.051	0.049	0.042	0.033	0.019	0.009	0.002
47	-0.075	-0.073	-0.064	-0.054	-0.032	-0.015	-0.004
48	0.490	0.478	0.430	0.368	0.243	0.125	0.035
74	0.476	0.463	0.413	0.350	0.226	0.113	0.031
84	-0.198	-0.187	-0.148	-0.110	-0.056	-0.023	-0.006
121	-0.009	-0.008	-0.008	-0.007	-0.005	-0.003	-0.001
125	0.772	0.751	0.664	0.555	0.339	0.153	0.035
141	0.172	0.166	0.144	0.117	0.070	0.032	0.008
143	-2.040	-1.483	-0.872	-0.568	-0.263	-0.106	-0.025
152	-0.007	-0.006	-0.005	-0.003	-0.002	-0.001	-0.000
153	0.008	0.008	0.008	0.008	0.006	0.003	0.001
157	-0.001	-0.001	-0.001	-0.001	-0.000	-0.000	-0.000
158	0.575	0.560	0.497	0.418	0.264	0.127	0.031
184	0.617	0.611	0.579	0.530	0.399	0.237	0.076
224	-0.019	-0.018	-0.015	-0.012	-0.006	-0.003	-0.001
250	-0.003	-0.003	-0.003	-0.002	-0.002	-0.001	-0.000
total	-6.169	-4.730	-2.161	-0.715	0.366	0.447	0.181
alpha	0.300	0.300	0.350	0.400	0.700	1.000	1.000
lod2	0.511	0.496	0.452	0.426	0.446	0.447	0.181

Table 4.3a Two-point lod scores between disease and marker for the **SLC1A5** locus at specific values of the recombination fraction (theta) using the **core schizophrenia** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-2.153	-1.820	-1.238	-0.812	-0.299	-0.048	0.039
27	-3.503	-2.992	-2.055	-1.408	-0.649	-0.246	-0.054
35	-2.355	-2.031	-1.366	-0.924	-0.432	-0.172	-0.040
36	-2.432	-2.078	-1.444	-1.011	-0.492	-0.201	-0.048
40	-2.236	-1.715	-1.064	-0.707	-0.328	-0.130	-0.030
41	-1.638	-1.300	-0.791	-0.522	-0.262	-0.136	-0.056
45	-0.100	-0.095	-0.078	-0.060	-0.032	-0.014	-0.003
46	-0.322	-0.304	-0.240	-0.178	-0.091	-0.038	-0.009
47	-0.100	-0.103	-0.111	-0.109	-0.078	-0.039	-0.010
48	-1.441	-1.128	-0.658	-0.416	-0.185	-0.073	-0.017
74	-1.665	-0.943	-0.367	-0.145	0.008	0.035	0.017
84	0.627	0.611	0.547	0.468	0.312	0.173	0.065
121	-0.006	-0.006	-0.005	-0.004	-0.002	-0.001	-0.000
125	-0.307	-0.288	-0.221	-0.158	-0.075	-0.029	-0.007
141	-0.185	-0.178	-0.151	-0.117	-0.059	-0.022	-0.005
143	-0.053	-0.051	-0.040	-0.026	-0.005	-0.004	-0.012
152	-0.026	-0.025	-0.021	-0.016	-0.009	-0.004	-0.001
153	-0.084	-0.081	-0.068	-0.053	-0.030	-0.013	-0.003
157	-0.023	-0.022	-0.018	-0.013	-0.007	-0.003	-0.001
158	-0.977	-0.648	-0.225	-0.060	0.024	0.018	0.001
184	-1.628	-1.175	-0.630	-0.372	-0.160	-0.089	-0.045
224	-0.030	-0.029	-0.025	-0.021	-0.012	-0.006	-0.001
250	0.043	0.044	0.046	0.043	0.030	0.015	0.004
total	-20.593	-16.355	-10.225	-6.620	-2.833	-1.028	-0.218
alpha	0.000	0.000	0.000	0.000	0.000	0.000	0.000
lod2	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 4.3b Two-point lod scores between disease and marker for the **SLC1A5** locus at specific values of the recombination fraction (theta) with **schizophrenia spectrum** model.

theta	0.000	0.010	0.050	0.100	0.200	0.300	0.400
cM	0.000	1.000	5.017	10.137	21.182	34.657	54.931
pedigree							
20	-1.964	-1.726	-1.204	-0.789	-0.284	-0.038	0.044
27	-4.723	-4.201	-2.899	-1.972	-0.916	-0.354	-0.077
35	-2.239	-1.976	-1.369	-0.938	-0.444	-0.178	-0.042
36	-1.670	-1.431	-0.952	-0.644	-0.305	-0.123	-0.029
40	-2.131	-1.716	-1.097	-0.735	-0.343	-0.136	-0.032
41	-2.194	-2.015	-1.369	-0.874	-0.364	-0.134	-0.035
45	0.023	0.022	0.019	0.015	0.009	0.004	0.001
46	0.278	0.269	0.237	0.197	0.121	0.058	0.015
47	-0.127	-0.130	-0.136	-0.130	-0.090	-0.044	-0.011
48	-1.236	-1.020	-0.624	-0.400	-0.179	-0.071	-0.017
74	-1.610	-0.923	-0.354	-0.134	0.015	0.039	0.018
84	0.422	0.409	0.359	0.299	0.188	0.098	0.035
121	-0.010	-0.010	-0.008	-0.007	-0.004	-0.002	-0.000
125	-0.230	-0.217	-0.170	-0.124	-0.060	-0.024	-0.005
141	-0.996	-0.899	-0.620	-0.402	-0.163	-0.053	-0.009
143	-0.242	-0.026	0.300	0.413	0.392	0.248	0.079
152	0.464	0.453	0.408	0.351	0.236	0.128	0.042
153	0.415	0.402	0.349	0.284	0.165	0.072	0.017
157	-0.012	-0.011	-0.009	-0.007	-0.004	-0.001	-0.000
158	-0.640	-0.471	-0.170	-0.034	0.034	0.023	0.003
184	-4.825	-4.255	-2.934	-2.096	-1.234	-0.788	-0.399
224	-0.257	-0.253	-0.231	-0.192	-0.109	-0.046	-0.011
250	0.014	0.015	0.018	0.018	0.014	0.007	0.002
total	-23.491	-19.710	-12.457	-7.902	-3.326	-1.314	-0.411
alpha	0.000	0.000	0.000	0.000	0.000	0.000	0.000
lod2	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 4.4 Results of extended relative pair analysis, ERPA, between schizophrenia and the glutamate receptor/transporter gene loci, GluR5, GluR6, and SLC1A5 respectively, showing expected and observed numbers of alleles shared identity-by-descent (IBD) between pairs of affected relatives with core schizophrenia model.

GluR5

	Alleles shared	Alleles Unshared
Expected	114.8	138.2
Observed	118.9*	134.1

Chi-sq $X^2 = 0.086$, df = 1

* Nonsignificant difference from the expected result

GluR6

	Alleles shared	Alleles Unshared
Expected	89.9	106.9
Observed	94.2*	102.5

Chi-sq $X^2 = 0.385$, df = 1

* Nonsignificant difference from the expected result

SLC1A5

	Alleles shared	Alleles Unshared
Expected	106.7	127.5
Observed	118.9*	134.1

Chi-sq $X^2 = 1.638$, df = 1

* Nonsignificant difference from the expected result

4.4 Discussion

The classical lod score method of linkage analysis necessitates that the transmission model for a trait be specified. If the model is mis-specified then artefactually negative lod scores may be produced, especially close to marker loci, and between flanking markers. Besides, lod score methods are least reliable at small recombination fractions because phenocopies and non-penetrant carriers not allowed for in the transmission model tend to be counted as recombinants. Nonparametric methods deliberately discard available information concerning the pattern of segregation of disease and markers through pedigrees, and doing this inevitably loses power. Attempts have been made to make lod score analysis less model-dependent by performing multiple analyses, by treating unaffected subjects as of unknown affection status and by ignoring multipoint data, but again these techniques inevitably reduce power. The MFLINK method described avoids having to rely on the confounding of recombination and transmission model parameters, and seeks to test for a genetic effect at a particular test position without making prior assumptions concerning the mode of transmission.

The results listed in section 4.1-4.3 suggest that allelic variation at GluR5, GluR6, and SLC1A5 do not provide a major gene contribution to schizophrenia susceptibility in a large proportion of these families. Because ERPA and model-free analyses were also performed, the negative results obtained are unlikely to be an artefact of an erroneous transmission model.

Although all methods of analysis produced results that weakly supported linkage between GluR6 and schizophrenia under admixture, it must be made clear that these results did not approach statistical significance and could easily have appeared by chance. A simulated lod analysis was further performed for the core schizophrenia model under the assumption that only 30% of these 23 pedigrees are linked to a single major locus. The simulation indicates that under the hypothesis of locus heterogeneity the mean lod at 0.00 recombination with GluR6 is 1.8, and the overall power to obtain a lod score over 1.00 is 61%, to obtain a lod score over 2.00 is 39%, and to obtain a lod over 3.00 is 22%. These results do seem more negative than would be expected if 30% of families were linked.

However, we cannot rule out the possibility that GluR5, GluR6, or SLC1A5 do exert an effect in some other families from other ethnic groups due to genetic heterogeneity in the aetiology of schizophrenia. It would be recommended that these loci be studied further in other datasets using methods designed to detect linkage and linkage disequilibrium, or both.

CHAPTER 5: GENE EXPRESSION STUDIES OF THE GLUTAMATE RECEPTOR SUBUNITS FOLLOWING TREATMENT WITH TRANS- AND CIS-FLUPENTHIXOL ANTIPSYCHOTIC DRUGS IN THE RAT BRAIN

5.1 Results of the gene expression studies on the NMDA receptor subunit genes

A representative autoradiograph of the multiprobe oligonucleotide solution hybridization (MOSH) result of the NMDA receptor subunit genes is shown in Figure 5.1. The 'blank' experiment, in which 20 ug of t-RNA instead of brain RNA was added in the hybridization buffer for the MOSH assay, showed no band (lane 'B') because all the unhybridized probes were digested by the S1 nuclease. The other lanes loaded with 20 ug of brain RNA showed multiple protected bands which were derived from the protected RNA:probe hybrid following the S1 enzyme digestion.

Long-term (24 week) treatment with either trans- or cis-flupenthixol significantly decreased the NMDAR1 mRNA levels in frontal cortex (Table 5.1). This flupenthixol-induced decrease in NR1 mRNA was observed at one week with trans- flupenthixol but was not shown in the cis-isomer treated groups until 24 weeks. A similar pattern of alteration was also noted in the cerebellum with the exception that the cis-drug was found not to change NR1 mRNA level at 24 weeks. In the subcortical structures, which is an area with abundant dopaminergic innervation such as the striatum and nucleus accumbens, trans-flupenthixol decreased NR1 mRNA levels after short-term (1 and 2 weeks) and long-term (12 and 24 weeks) but not subchronic (4 and 8 weeks) treatment. Cis-flupenthixol decreased NR1 mRNA expression only after 12 weeks of drug treatment. Trans-flupenthixol also decreased NR1 mRNA in the parietal cortex throughout all the time points we examined in contrast to the cis-drug which decreased it at 2 and 4 weeks (Table 5.1).

Both trans- and cis-flupenthixol decreased the NMDAR2A gene mRNA

expression in all the four brain regions tested at 24 weeks with the exception that cis-flupenthixol did not affect NR2A mRNA expression level in the subcortical region ($p=0.918$). In the cerebellar and subcortical regions trans-drug also decreased NR2A mRNA at 1 and 12 weeks in contrast to cis-drug which decreased it at 12 weeks (Table 5.2).

NMDAR2B mRNA is relatively more abundant than the other NMDA receptor subunit genes and its regulation by flupenthixol appears to show a biphasic response. Both trans- and cis-flupenthixol increased the NR2B mRNA level after short-term (1 or 2 weeks) treatment whereas its expression was significantly reduced after long-term (12 or 24 weeks) treatment in the cerebellar and subcortical areas; a similar pattern was also found in the frontal cortex of the cis-flupenthixol treated brains. However, trans-flupenthixol decreased NR2B receptor gene mRNA expression at all the time points we examined in the parietal cortex whereas NR2B mRNA was increased with cis-isomer at only the week one time point (Table 5.3).

The NMDAR2C gene mRNA expression was elevated first at weeks 1 or 2 then decreased after treatment with cis-flupenthixol at the 12 or 24 week time points in all four brain regions. This pattern was also found in the cerebellar and subcortical regions in response to the trans-isomer. In the parietal cortex, trans-isomer decreased the NR2C mRNA after short-term (1, 2 weeks) or long-term (12, 24 weeks) treatment. (Table 5.4).

The NMDAR2D gene mRNA expression in the frontal cortex was not affected at any time point by either isomer. Its expression was elevated in the cerebellar and subcortical areas after 1 to 8 weeks of treatment with flupenthixol then either returned to normal or decreased at 12 and 24 weeks. (Table 5.5).

Table 5.6 summarises the alteration of the five NMDA receptor subunit mRNA levels following treatment with either trans- or cis- flupenthixol. Generally speaking, flupenthixol, especially its trans-isoform, significantly decreased the mRNA expression of NR1 in most brain areas we tested. NR2B and NR2C mRNAs were first increased after treatment with flupenthixol for 1 to 8 weeks then decreased after long-term (12, 24 weeks) treatment in the subcortical region. NR2A and 2D, which are the two relatively less abundant NMDA receptor subunits, are also relatively unaffected by flupenthixol in terms of their mRNA expression. However,

NR2A mRNA was significantly decreased compared to controls in most brain areas after 24 weeks of treatment.

Immunoblot analysis of brain membrane preparations from four separate regions of right brain shows labelling of NMDAR1 as a single immunoreactive band with an estimated mass of 116 kDa (Fig. 5.2). Two time points, 4 weeks and 24 weeks, of drug-treatment were examined because it is generally accepted that flupenthixol reaches the maximal therapeutic efficacy after 3-4 weeks of initial administration (Johnstone et al., 1978). The time-point of 24 weeks is to test the long-term effect of this drug. Both trans- and cis-flupenthixol significantly reduced the immunoreactivity of NR1 in right cerebellum after 24 weeks of treatment (Fig. 5.2 and Table 5.7). Four weeks of treatment with cis-flupenthixol also decreased the NR1 subunit protein level in the right parietal cortex about 17% compared with the corresponding control group (Table 5.7). However, it did not reach a statistically significant level (P=0.12, Wilcoxon's rank sum test).

5.2 Results of the gene expression studies on the AMPA receptor subunit genes

Figure 5.3 shows a representative autoradiograph of the multiprobe oligonucleotide solution hybridization (MOSH) results of the AMPA subtype of glutamate receptor subunit genes (GluR1, 2, 3, 4) and D_2 dopamine receptor gene. Similar to that of NMDA receptor subunit genes shown in Fig. 5.1, the 'blank' experiment, in which 20 ug of t-RNA instead of brain RNA was added in the hybridization buffer for the MOSH assay, showed no band (lane 'B') because all the unhybridized probes were digested by the S1 nuclease. The other lanes loaded with 20 ug of brain RNA showed multiple protected bands which were derived from the protected RNA:probe hybrid following the S1 enzyme digestion.

The mRNA levels of the AMPA type of glutamate receptor subunit, GluR1-4, following 4, 12, and 24 week treatment with trans- or cis-flupenthixol are summarized in Table 5.8 - 5.11. No significant changes were observed.

Western immunoblot analysis using antibodies against GluR2/3 produced immunoreactive bands of approximately 105 kDa (Fig. 5.4). The Western blotting

results for the GluR2/3 subunit immunoreactivity in the right rat brain following 4 or 24 week treatment with trans- and cis- flupenthixol are summarized in Table 5.12. Levels of GluR2/3 subunit immunoreactivity did not differ between control and either trans- or cis- flupenthixol treated rats in any of the four regions examined.

The mRNA levels of the dopamine D_2 receptor gene following 4, 12, 24 week treatment with trans- or cis-flupenthixol are summarized in Table 5.13. Cis-flupenthixol upregulated the D_2 receptor mRNA expression up to nearly two fold (12 weeks, subcortical region) compared with the control rats. The increase in D_2 receptor mRNA returned to normal levels after 24 weeks except in the cerebellum. Trans-flupenthixol also significantly upregulated D_2 receptor mRNA expression in subcortical regions after 12 weeks of treatment with a 30% increase.

5.3 Results of the gene expression studies on the kainate receptor subunit genes

A representative autoradiograph of the multiprobe oligonucleotide solution hybridization (MOSH) results of the kainate subtype of glutamate receptor subunit genes (GluR5, 6, 7, KA1, and KA2) is shown in Figure 5.5. The mRNA levels of the kainate type of glutamate receptor subunit following 4 and 24 weeks of treatment with trans- or cis-flupenthixol are summarized in Table 5.14 - 5.18. No significant change of any gene was observed.

Western blot analysis using polyclonal antibodies against combined GluR6/7 glutamate receptor subunits produced immunoreactive bands of approximately 115 kDa (Fig. 5.6). The Western (immuno) blotting results for the GluR6/7 subunit immunoreactivity in the right rat brain following 4 or 24 week treatment with trans- and cis- flupenthixol are summarized in Table 5.19. Levels of GluR6/7 subunit immunoreactivity did not differ between control and either trans- or cis- flupenthixol treated rats in any of the four regions examined.

Table 5.1 NR1 NMDA receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	1 week	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>						
Frontal cortex	80.9± 3.7**	86.4± 6.1*	86.4± 3.5*	88.6± 3.6*	63.0± 5.2**	628± 25***
Cerebellum	68.6± 4.6**	70.1± 3.0**	95.4± 5.1	52.5± 4.7***	86.7± 6.0*	709± 28**
Subcortical structures	50.2± 9.1***	65.8± 9.4*	96.7± 4.4	90.1± 8.6	47.6± 2.9***	575± 44***
Parietal cortex	61.0± 2.0***	43.3± 3.2***	86.5± 9.7*	88.0± 4.1*	45.3± 3.2**	809± 24**
<u>cis-flupenthixol:</u>						
Frontal cortex	90.5± 3.0	87.7± 9.2	91.3±11.0	91.9± 4.4	98.2±15.0	690± 24***
Cerebellum	96.5± 8.1	98.1± 4.8	92.4± 5.0	84.0± 4.2	101.2± 5.1	88.0± 4.1
Subcortical structures	119.6±15.0	87.0± 4.7	106.5± 4.4	109.4± 6.4	22.6± 2.0***	91.4± 7.5
Parietal cortex	88.8±10.1	70.2± 3.6**	78.3± 4.2**	96.3± 2.1	115.2± 8.0	99.6± 6.6

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)
* Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon's rank sum test)

Table 5.2 NR2A NMDA receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	1 week	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>						
Frontal cortex	78.9±10.1	107.3±15.4	90.7± 4.9	93.1±16.4	79.6± 4.9*	60.9± 9.1**
Cerebellum	62.9± 9.7*	97.6± 9.8	111.3± 5.9	102.8±21.3	67.2± 9.2**	58.2±11.9*
Subcortical structures	46.1± 8.4**	92.5± 4.6	95.0± 5.7	78.3±19.1	46.2± 3.6***	32.4± 6.6***
Parietal cortex	47.0± 4.4***	81.3±18.6	93.1±11.5	82.4±13.4	36.1± 5.0*	79.1± 7.0**
<u>cis-flupenthixol:</u>						
Frontal cortex	93.5± 6.1	112.4± 9.8	93.4± 5.4	94.9± 8.1	105.9±18.6	49.6± 4.2**
Cerebellum	96.7±10.1	93.2± 7.2	98.9± 7.5	88.8±14.3	77.2± 5.4*	24.8± 2.6***
Subcortical structures	146.5±21.4	96.8±10.5	110.9± 4.6	80.0±15.4	21.5± 3.3***	109.1±15.6
Parietal cortex	76.4±12.2	89.6± 3.6	86.6± 5.3	93.0± 5.9	95.4±10.6	74.8± 4.0**

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)
* Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon's rank sum test)

Table 5.3 NR2B NMDA receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	1 week	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
trans-flupenthixol:						
Frontal cortex	91.2± 5.2	162.8±14.8*	90.3± 2.9	86.7± 6.6	87.3±12.5	88.7± 3.2
Cerebellum	166.1±29.9*	165.4±31.8*	111.8± 5.8	116.8±15.1	67.1± 7.7*	105.7±14.2
Subcortical structures	90.7± 8.4	120.0± 9.1*	100.3± 3.5	90.3±11.1	64.6± 2.2***	63.3± 5.2**
Parietal cortex	87.6± 2.0**	73.0± 7.0*	78.9± 7.1*	79.0± 4.2*	81.0± 7.0	85.4± 4.8*
cis-flupenthixol:						
Frontal cortex	101.2± 4.6	193.4± 9.2***	94.1± 3.2	81.0± 3.3*	90.1± 8.3	72.3± 5.9***
Cerebellum	122.2± 9.3*	243.0±47.6***	99.0± 7.5	97.3±12.9	67.6± 3.9*	76.3±11.5*
Subcortical structures	145.4±13.4*	185.9± 8.4***	114.7± 2.5*	153.8± 8.1**	55.6± 4.1***	80.6± 5.4*
Parietal cortex	137.5±15.9*	118.2± 6.2	86.9± 9.0	91.6± 1.4	93.1±14.0	90.4± 2.9

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)
* Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon's rank sum test)

Table 5.4 NR2C NMDA receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	1 week	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
trans-flupenthixol:						
Frontal cortex	83.6± 5.9	121.5±14.2	92.7± 4.8	113.7±17.7	152.6±14.1*	84.9± 4.4
Cerebellum	138.6±19.3	110.3± 6.5	97.0± 4.9	82.5± 7.1	75.1± 3.8**	77.0± 9.6
Subcortical structures	82.9±10.2	141.9±14.8*	97.9± 5.0	127.2±19.1	61.3± 3.0***	45.2± 4.8***
Parietal cortex	68.9± 3.9*	71.0±12.7*	89.1± 9.3	92.2±11.3	66.0± 8.7*	78.7± 3.8**
cis-flupenthixol:						
Frontal cortex	91.1± 5.9	118.2± 7.6*	96.5± 4.6	99.7± 7.0	144.3±20.0	68.4± 4.9*
Cerebellum	111.4±11.1*	143.2±11.9*	93.8± 6.1	89.6± 6.7	76.1± 2.4**	34.2± 1.8***
Subcortical structures	139.7±12.7*	245.7± 8.9***	203.9±21.0*	204.4±11.9***	57.9± 4.3***	104.1±11.7
Parietal cortex	140.6±20.9	189.3±21.4**	89.9± 4.5	97.6± 3.9	87.1±12.1	75.4± 2.8***

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)
* Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon's rank sum test)

Table 5.5 NR2D NMDA receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	1 week	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>						
Frontal cortex	83.3±11.7	147.5±29.3	104.1± 5.0	97.2±17.1	108.5±13.3	94.3± 3.5
Cerebellum	160.5±24.3	161.4±30.7	128.6± 8.9	145.5±20.3	79.5± 8.3	71.8± 8.5
Subcortical structures	77.5±12.5	230.4±36.3**	113.7± 4.8	148.9±21.4	65.3± 3.3***	45.5± 6.3**
Parietal cortex	50.9± 3.7***	131.2±14.1	98.4± 9.7	93.3±15.0	72.6±10.2	53.7± 3.5**
<u>cis-flupenthixol:</u>						
Frontal cortex	108.2±12.1	87.7± 8.8	92.3± 6.1	84.2± 7.0	103.5± 7.0	94.0± 3.5
Cerebellum	142.5± 6.6***	242.8±30.7**	99.8± 7.3	103.4±15.4	67.2± 4.8**	87.7±20.9
Subcortical structures	145.8±23.0	222.0± 9.0***	120.9± 3.9**	217.5±17.0***	85.0± 9.4	89.6±11.1
Parietal cortex	65.4± 8.4*	84.5±22.0	88.9± 6.9	98.0± 5.2	95.3±15.4	88.8±11.2

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)
* Significantly different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon's rank sum test)

Table 5.6 Summary of the NMDA receptor subunit mRNA level changes following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day). ↑: increase; ↓: decrease; =: no change.

Week of treatment		1	2	4	8	12	24
<u>trans-flupenthixol:</u>							
Frontal cortex	NR1	↓	↓	↓	↓	↓	↓
	NR2A	=	=	=	=	↓	↓
	NR2B	=	↑	=	=	=	=
	NR2C	=	=	=	=	↑	=
	NR2D	=	=	=	=	=	=
Cerebellum	NR1	↓	↓	=	↓	↓	↓
	NR2A	↓	=	=	=	↓	↓
	NR2B	↑	↑	=	=	↓	=
	NR2C	=	=	=	=	↓	=
	NR2D	=	=	=	=	=	=
Subcortical structures	NR1	↓	↓	=	=	↓	↓
	NR2A	↓	=	=	=	↓	↓
	NR2B	=	↑	=	=	↓	↓
	NR2C	=	↑	=	=	↓	↓
	NR2D	=	↑	=	=	↓	↓
Parietal cortex	NR1	↓	↓	↓	↓	↓	↓
	NR2A	↓	=	=	=	↓	↓
	NR2B	↓	↓	↓	↓	=	↓
	NR2C	↓	↓	=	=	↓	↓
	NR2D	↓	=	=	=	=	↓
<u>cis-flupenthixol:</u>							
Frontal cortex	NR1	=	=	=	=	=	↓
	NR2A	=	=	=	=	=	↓
	NR2B	=	↑	=	↓	=	↓
	NR2C	=	↑	=	=	=	↓
	NR2D	=	=	=	=	=	=
Cerebellum	NR1	=	=	=	=	=	=
	NR2A	=	=	=	=	↓	↓
	NR2B	↑	↑	=	=	↓	↓
	NR2C	↑	↑	=	=	↓	↓
	NR2D	=	=	=	=	↓	=

Table 5.6 (continued)

Subcortical structures	NR1	=	=	=	=	↓	=
	NR2A	=	=	=	=	↓	=
	NR2B	↑	↑	↑	↑	↓	↓
	NR2C	↑	↑	↑	↑	↓	=
	NR2D	=	↑	↑	↑	=	=
Parietal cortex	NR1	=	↓	↓	=	=	=
	NR2A	=	=	=	=	=	↓
	NR2B	↑	=	=	=	=	=
	NR2C	=	↑	=	=	=	↓
	NR2D	↑	=	=	=	=	=

In Table 5.6, data of significant increase or decrease were derived from Table 5.1 - 5.5 at a significance level of 0.05 (i.e., $p < 0.05$) without considering the possible false positive findings by chance. Among the 240 Wilcoxon's test results, 111 were considered as having a significant difference at the level of 0.05 and 72 were significant at the level of 0.01. If a correction for multiple testing is applied, 12 results would have been found positive by chance at the significance level of 0.05 whilst 2.4 results would be positive by chance at the significance level of 0.01. Therefore, few of the significant results obtained would have been caused by multiple testing. Applying a Bonferroni correction for multiple testing would be excessively conservative for the non-independent measures of the MOSH quantification. Nevertheless if correction for 48 tests is applied to the most significant main NMDAR1 results ($p < 0.048$), then the result is still significant.

Table 5.7 NR1 NMDA receptor subunit immunoreactivity following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	97.4±11.2	101.3±14.4
Cerebellum	92.9± 5.4	63.1±15.2*
Subcortical structures	104.2±16.0	97.0± 8.0
Parietal cortex	99.7±14.5	88.0±11.7
<u>cis-flupenthixol:</u>		
Frontal cortex	100.4±10.5	105.2±12.2
Cerebellum	94.3±12.7	78.0±14.1*
Subcortical structures	106.4±13.9	96.0±11.0
Parietal cortex	83.0±15.9	88.5±12.0

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

: Significantly different from control ($p < 0.05$, Wilcoxon's rank sum test)

Table 5.8 GluR1 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>			
Frontal cortex	97.0± 4.5	97.5± 5.9	95.2± 8.4
Cerebellum	101.9± 2.4	89.8± 6.6	111.6±15.9
Subcortical structures	91.2± 8.3	91.5± 5.4	90.8± 8.9
Parietal cortex	101.3±10.0	91.0± 5.4	93.9± 9.0
<u>cis-flupenthixol:</u>			
Frontal cortex	112.0± 6.2	112.0± 6.5	117.7±12.3
Cerebellum	102.2± 3.6	103.2± 8.4	99.0±14.1
Subcortical structures	102.9± 5.6	106.0± 5.0	91.7± 9.1
Parietal cortex	105.2±10.1	106.2±10.9	103.4± 8.4

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.20$, Wilcoxon's rank sum test)

Table 5.9 GluR2 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>			
Frontal cortex	92.6± 2.3	97.4± 6.6	103.9± 3.6
Cerebellum	97.3± 2.2	85.3± 5.2	101.8± 8.9
Subcortical structures	86.8± 5.8	94.0± 5.8	93.3± 7.0
Parietal cortex	98.4± 9.5	98.4± 9.6	94.5± 7.1
<u>cis-flupenthixol:</u>			
Frontal cortex	109.5± 5.0	103.0± 8.1	115.4± 4.9
Cerebellum	100.3± 2.4	89.5± 7.2	103.8±13.4
Subcortical structures	102.2± 6.1	107.5± 3.8	98.7± 7.0
Parietal cortex	111.3± 11.5	110.3± 4.4	98.1± 7.2

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.15$, Wilcoxon's rank sum test)

Table 5.10 GluR3 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>			
Frontal cortex	90.6± 9.2	93.4± 9.9	89.1± 3.6
Cerebellum	94.0± 4.5	89.5± 7.0	87.4± 7.7
Subcortical structures	98.7±12.5	93.3± 6.3	89.1±10.2
Parietal cortex	109.0±10.0	109.0±12.0	92.1± 5.2
<u>cis-flupenthixol:</u>			
Frontal cortex	113.7± 5.4	99.6± 9.5	100.2± 4.7
Cerebellum	93.6± 3.8	98.7± 9.3	81.5±11.4
Subcortical structures	99.6±11.5	107.2± 3.2	91.3±10.9
Parietal cortex	111.4± 5.5	111.3± 5.2	97.4± 9.9

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.10$, Wilcoxon's rank sum test)

Table 5.11 GluR4 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>			
Frontal cortex	94.5± 8.2	85.0±11.7	89.3± 3.7
Cerebellum	91.9± 4.0	88.6± 7.2	96.0± 7.5
Subcortical structures	110.4±11.0	87.7± 8.0	90.6± 9.1
Parietal cortex	112.4±11.0	112.4±10.0	98.7± 5.7
<u>cis-flupenthixol:</u>			
Frontal cortex	110.6± 5.9	94.9±10.0	98.7± 4.2
Cerebellum	95.1± 2.4	89.4± 3.7	90.4± 7.0
Subcortical structures	105.9± 8.4	108.3± 3.5	107.7±11.3
Parietal cortex	106.0± 8.5	105.9± 8.5	106.9± 4.3

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.10$, Wilcoxon's rank sum test)

Table 5.12 GluR2/3 AMPA receptor subunit immunoreactivity following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	95.0±12.9	106.9±12.7
Cerebellum	85.5±12.2	86.2±10.0
Subcortical structures	115.4± 8.4	102.5±10.7
Parietal cortex	115.2±10.8	90.0± 8.3
<u>cis-flupenthixol:</u>		
Frontal cortex	94.0±10.7	111.9±10.9
Cerebellum	96.2±10.2	87.1±15.1
Subcortical structures	110.4± 9.9	100.9± 8.4
Parietal cortex	95.7± 9.2	94.6± 8.6

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.20$, Wilcoxon's rank sum test)

Table 5.13 **D2** dopamine receptor mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	12 weeks	24 weeks
<u>trans-flupenthixol:</u>			
Frontal cortex	104.4± 9.8	92.0±12.9	91.7±12.3
Cerebellum	124.6± 7.5	127.2±10.2	141.4±14.7*
Subcortical structures	112.3±12.0	135.5± 4.4***	90.5±11.4
Parietal cortex	N/A	N/A	N/A
<u>cis-flupenthixol:</u>			
Frontal cortex	126.5± 7.9*	129.4±10.7*	102.0±13.0
Cerebellum	136.1± 4.9**	175.5±13.9*	146.8±13.3*
Subcortical structures	105.7± 8.1	194.5± 3.4***	105.8±14.1
Parietal cortex	N/A	N/A	N/A

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

* Significantly different from control, *p < 0.05 **P < 0.01 ***P < 0.001 (Wilcoxon's rank sum test)

N/A: not available

Table 5.14 GluR5 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	N/A	N/A
Cerebellum	100.0±13.0	99.5± 4.3
Subcortical structures	N/A	103.5± 6.4
Parietal cortex	90.0±12.1	N/A
<u>cis-flupenthixol:</u>		
Frontal cortex	N/A	N/A
Cerebellum	106.4±11.0	101.0± 8.2
Subcortical structures	N/A	97.4± 8.0
Parietal cortex	89.4± 7.1	N/A

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.25$, Wilcoxon's rank sum test)

N/A: not available

Table 5.15 GluR6 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	105.5±12.5	91.8± 4.2
Cerebellum	92.5± 5.0	105.3± 3.3
Subcortical structures	90.5± 4.9	100.7± 4.5
Parietal cortex	87.4± 6.0	95.9± 3.0
<u>cis-flupenthixol:</u>		
Frontal cortex	100.5±11.7	94.7± 4.9
Cerebellum	90.2± 6.2	102.5± 5.3
Subcortical structures	94.3± 6.9	99.3± 4.7
Parietal cortex	96.1±13.0	115.9± 9.3

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.15$, Wilcoxon's rank sum test)

Table 5.16 GluR7 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	98.9± 7.1	90.5± 5.0
Cerebellum	89.0± 5.0	98.7± 3.8
Subcortical structures	99.3± 3.5	96.9± 4.3
Parietal cortex	103.5± 7.4	89.9± 4.4
<u>cis-flupenthixol:</u>		
Frontal cortex	99.0± 9.9	95.9± 2.6
Cerebellum	89.4± 5.0	98.3± 3.3
Subcortical structures	108.1± 4.3	95.6± 4.0
Parietal cortex	91.1±10.1	97.5± 3.6

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.10$, Wilcoxon's rank sum test)

Table 5.17 KA1 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	117.0±16.5	97.3± 8.5
Cerebellum	95.5±17.0	113.5±12.5
Subcortical structures	88.9±11.3	107.0± 7.2
Parietal cortex	83.8±20.0	87.2± 9.3
<u>cis-flupenthixol:</u>		
Frontal cortex	103.9±15.0	105.4±11.9
Cerebellum	102.4±12.0	111.3±11.2
Subcortical structures	92.5±12.5	97.5± 8.0
Parietal cortex	104.9±19.5	103.4± 9.0

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

Not significantly different from control ($p > 0.10$, Wilcoxon's rank sum test)

Table 5.18 KA2 glutamate receptor subunit mRNA levels following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	109.5±15.5	102.7± 6.9
Cerebellum	94.1±14.1	97.0± 7.7
Subcortical structures	85.6±11.0	99.2±10.5
Parietal cortex	87.4±10.0	95.1±10.5
<u>cis-flupenthixol:</u>		
Frontal cortex	108.0±13.2	108.3±13.3
Cerebellum	97.0±15.2	99.3±10.2
Subcortical structures	97.2±14.1	92.7± 9.3
Parietal cortex[1]	102.9±16.0	91.6± 9.0

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

[1] Not significantly different from control ($p > 0.20$, Wilcoxon's rank sum test)

Table 5.19 GluR6/7 glutamate receptor subunit immunoreactivity following treatment with trans- or cis-flupenthixol (0.2/mg/kg/day)

	4 weeks	24 weeks
<u>trans-flupenthixol:</u>		
Frontal cortex	105.9± 9.1	102.3±15.0
Cerebellum	94.4± 9.1	88.0± 3.2
Subcortical structures	93.2± 5.0	104.2±11.3
Parietal cortex	113.0± 9.9	108.0±13.6
<u>cis-flupenthixol:</u>		
Frontal cortex	109.0± 4.4	97.5± 6.7
Cerebellum	97.5±17.3	100.4± 8.7
Subcortical structures	91.3± 7.5	102.5±10.9
Parietal cortex	108.9±12.3	95.9± 6.9

Data are expressed as percentage of corresponding control and are the mean ± SEM. (n=8/group)

*: Not significantly different from control ($p > 0.15$, Wilcoxon's rank sum test)

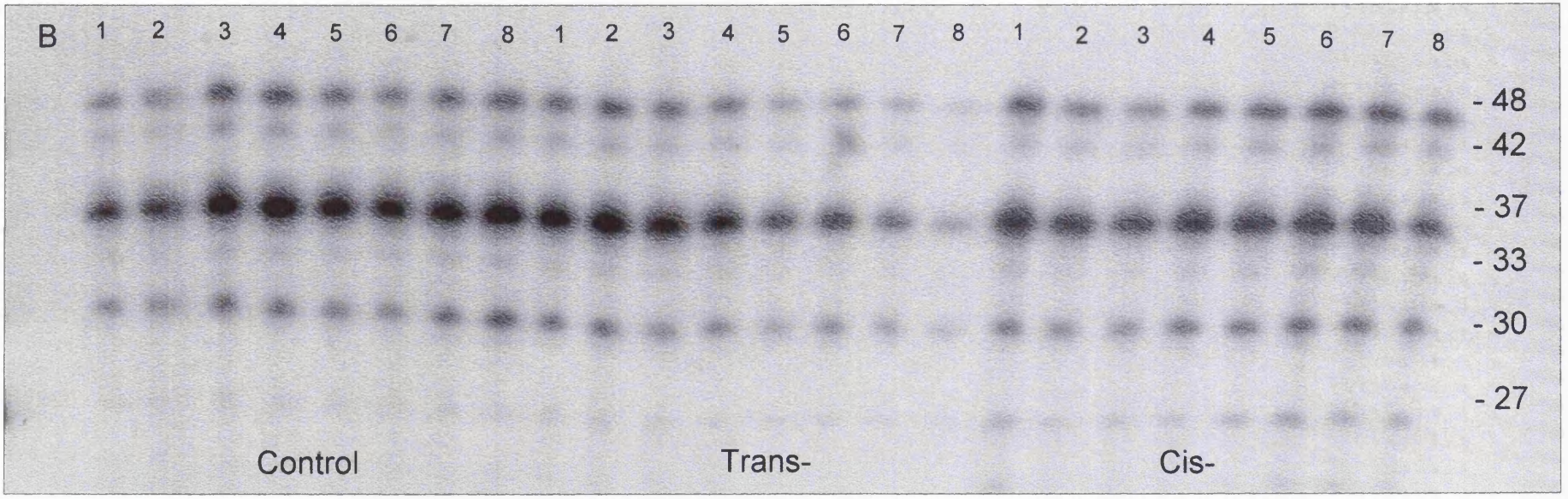

Figure 5.1 Representative autoradiograph (24 weeks of treatment in the parietal cortex) from the MOSH experiments of the NMDA receptor subunits. Each lane represents a different brain sample while lane 'B' is the 'blank experiment' in which 20 ug of t-RNA was used instead of brain RNA. The size of each probe used is as follows: NMDAR1: 48 mer, NMDAR2A: 42 mer, ß-actin: 37 mer, NMDAR2C: 33 mer, NMDAR2B: 30 mer, and NMDAR2D: 27 mer.

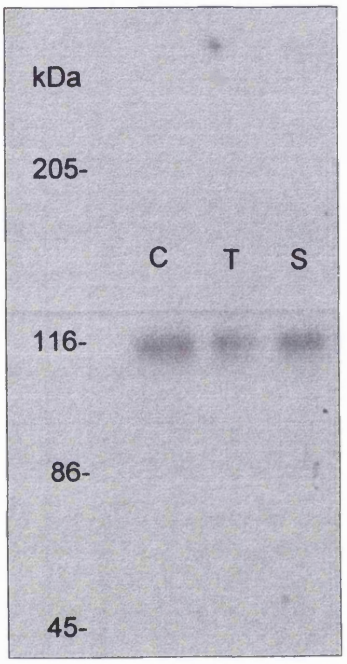

Figure 5.2 Immunoblot analysis of brain membrane preparations from the right cerebellum following 24 weeks of drug treatment shows labelling of NMDAR1 subunit as a single immunoreactive band (~116 kDa). C:control, T:trans-, S:cis-.

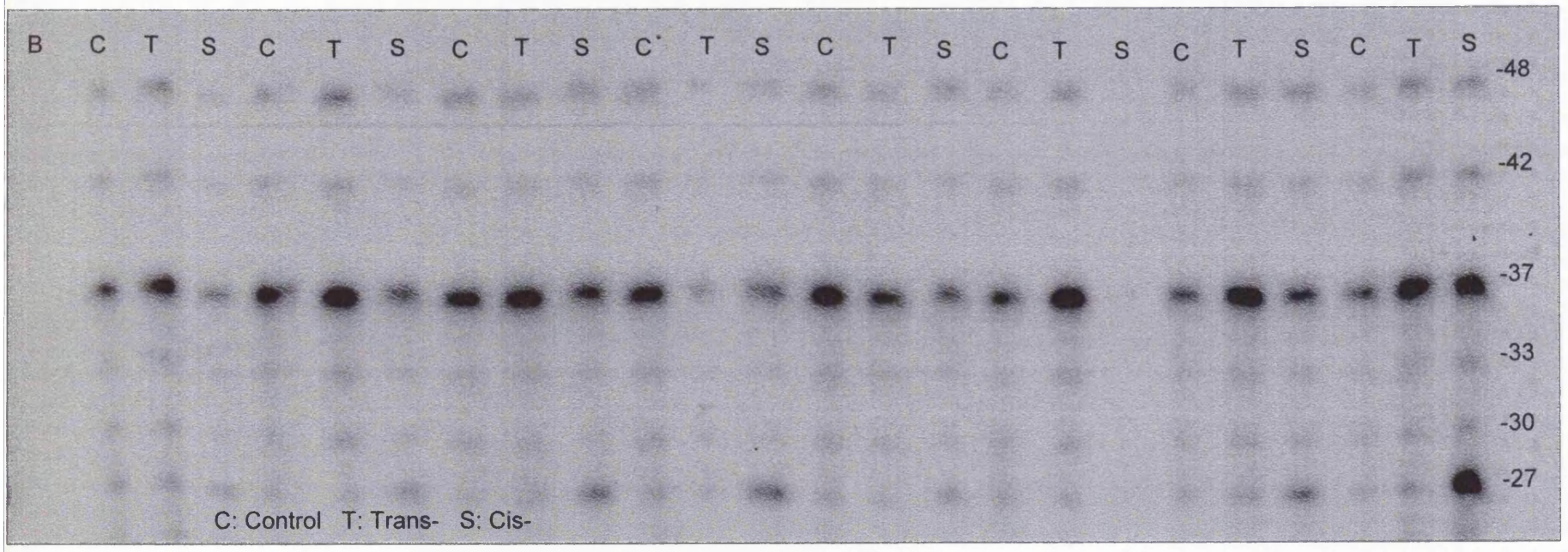

Figure 5.3 Representative autoradiograph (12 weeks of treatment in the subcortical region) from the MOSH experiments on the AMPA receptor subunits and D2 dopamine receptor. Each lane represents a different brain sample while lane 'B' is the 'blank experiment' in which 20 ug of t-RNA was used instead of brain RNA. The size of each probe used is as follows: GluR4: 48 mer, GluR3: 42 mer, ß-actin: 37 mer, GluR2: 33 mer, GluR1: 30 mer, and D2: 27 mer.

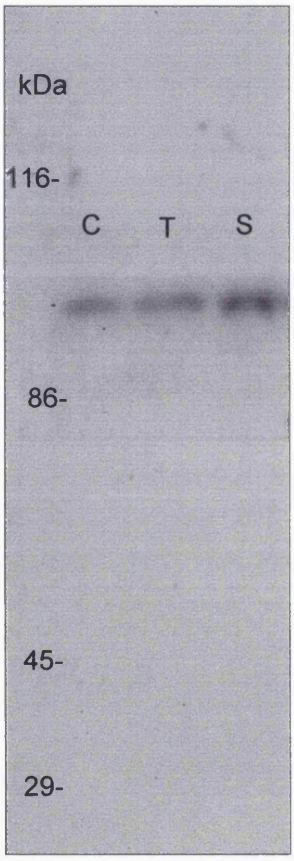

Figure 5.4 Representative immunoblot analysis of brain membrane preparations (the right subcortical region after 4 weeks of drug treatment) shows labelling of GluR2/3 subunit as a single immunoreactive band (~105 kDa). C:control, T:trans-, S:cis-.

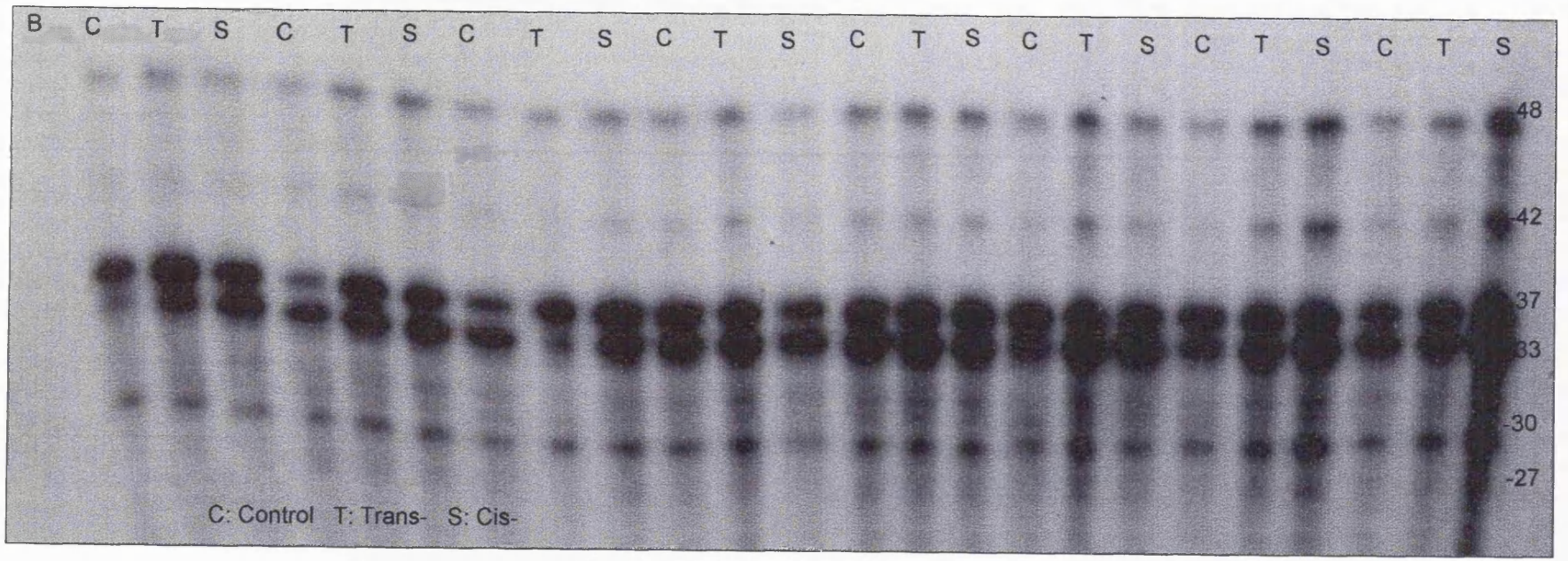

Figure 5.5 Representative autoradiograph (24 weeks of treatment in the frontal lobe region) from the MOSH experiments on the kainate receptor subunits. Each lane represents a different brain sample while lane 'B' is the 'blank experiment' in which 20 ug of t-RNA was used instead of brain RNA. The size of each probe used is as follows: KA2: 48 mer, KA1: 42 mer, ß-actin: 37 mer, GluR7: 33 mer, GluR6: 30 mer, and GluR5: 27 mer. The GluR5 (27 mer) mRNA levels were too low to be detected in this region.

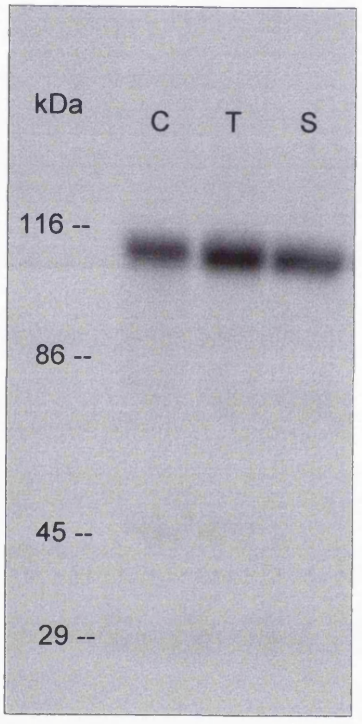

Figure 5.6 Representative immunoblot analysis of brain membrane preparations from the right brain (frontal lobe, 4 weeks of drug treatment) shows labelling of GluR6/7 subunit as a single immunoreactive band (~ 115 kDa). C:control, T:trans-, S:cis-.

5.4 Discussion

Several methods often used in determination of the abundance of specific mRNAs include Northern blot analysis, reverse transcription - polymerase chain reaction (RT-PCR), in situ hybridization, and solution hybridization either using riboprobes with RNase or antisense DNA probes with S1 enzyme (Sambrook et al., 1989). The multiprobe oligonucleotide solution hybridization (MOSH) method was chosen and developed for this study for quantification of the relative abundance of specific mRNAs in the light of several advantages with this technique, as follows: 1) Solution hybridization protocols, e.g., MOSH, are thought to be more sensitive to detect low abundance mRNAs, compared with the traditional Northern blot method that relies on RNA bound to a solid support (Buckland et al., 1992). 2) Synthetic antisense oligonucleotides can be employed as probes, i.e., there is no need to prepare longer probes such as riboprobes from clones. 3) Because the probes used in the S1 assay are significantly shorter than the mRNA species being detected, the target RNA preparation need not be completely intact. Breaks in mRNA that occur outside the region that hybridizes to the probe will have no effect on the S1 assay but will result in band smearing on Northern blots. 4) Multiple probes of different size can be used simultaneously in each experiment, i.e., multiple mRNA species are able to be detected simultaneously.

Using MOSH, Oretti et al. (1994) did not find any change of the levels of mRNA encoding the NMDA receptor subunits (NR1, NR2A, NR2B, and NR2C) in the whole rat brain following 1-32 days of haloperidol or sulpiride administration. However, using in situ hybridization technique, Meshul et al. (1996) found that subchronic (28 days) administration of haloperidol or clozapine significantly decreased the abundance of NMDAR1 subunit mRNA containing a 63-base insert in the rat caudate nucleus. Both drugs increased the mRNA levels for NMDAR2B, though only the increase caused by clozapine reached a statistically significant level. In addition, both drugs had no effect on ^{3}H-MK801 binding to striatal membrane preparations. These results are consistent with our findings that flupenthixol generally decreased the mRNA levels of NR1 in most brain region throughout 24 weeks of treatment, yet it first upregulated the mRNA levels of NR2B subunit

following subchronic treatment (2-8 weeks) then decreased the levels after long term treatment (24 weeks). The negative results reported by Oretti et al. (1994) could be due to the gross brain area (whole brain) instead of a smaller region being studied thus a tiny change in a fine area would be difficult to detect. In addition, the different pharmacological characteristics such as their affinities to several neuroreceptors between flupenthixol and other antipsychotic drugs may also contribute to the variations among different studies.

In contrast to NMDA receptor subunits, AMPA and kainate receptor subunits were not affected by long-term treatment of flupenthixol. These results were consistent with the negative findings from Oretti et al. (1994) in which GluR1-4 receptor mRNA levels were examined in the whole brain. Similarly consistent negative findings of the glutamate receptor binding in response to antipsychotic drugs were also reported by Tarazi et al. (1996) in which haloperidol, raclopride, and clozapine were all found to have no effect on the NMDA, AMPA, or kainate receptor binding after 28 days of treatment. The exception was that haloperidol decreased the NMDA receptor (MK-801) binding in the prefrontal cortex area only. With the exception of the KA1 subunit, all other non-NMDA receptor subunit mRNA levels were found differentially regulated by 14-day treatment of haloperidol or clozapine in the rat hippocampal formation (Meador-Woodruff et al., 1996). Again that could be due to the smaller regions chosen for this study. However, as mentioned earlier, generalizability of these findings from haloperidol and clozapine, the representative typical and atypical antipsychotic drugs respectively, to all antipsychotics is premature until the effects of other drugs on glutamate receptor subunit expression have been determined.

The mechanisms underlying the regulation of glutamate receptor subunit gene expression by the antipsychotic drugs and their clinical correlates will be discussed in section 6.2.

CHAPTER 6: GENERAL DISCUSSION

6.1 The feasibility of linkage studies in schizophrenia

Genetic studies of schizophrenia have a long history. The generally accepted model for the aetiology of schizophrenia is genetic variation interacting with non-genetic variation to determine variation in liability or susceptibility to the disease (Kidd, 1997). For the past decade, linkage analysis has been dramatically successful for Mendelian disorders but has been less successful than had been expected for complex traits or disorders such as heart diseases, hypertension, diabetes, infection, and many mental diseases including schizophrenia (Lander et al, 1994). It is rather difficult to find a genetic marker that shows perfect cosegregation with such "complex traits". The reasons for this can be ascribed to a few basic problems as follows:

1) Incomplete penetrance and phenocopy. "Incomplete penetrance" refers to the fact that some individuals who inherit a predisposing allele may not manifest the disease, whereas "phenocopy" refers to others who inherit no predisposing allele yet get the disease as a result of environmental or random causes. Thus, genetic mapping is hampered by the fact that a predisposing allele may be present in some unaffected individuals or absent in some affected individuals.

2) Genetic heterogeneity. Mutations in any one of several genes may result in identical phenotypes. Examples of genetic heterogeneity in human diseases include polycystic kidney disease (Reeders et al., 1987), early-onset Alzheimer's disease (St. George-Hyslop et al, 1990; 1994), maturity-onset diabetes of the young (Froguel et al., 1992), hereditary nonpolyposis colon cancer (Fishel et al., 1994), and ataxia telangiectasia (Sobel et al., 1992), etc. Medical geneticists are normally unable to know whether two patients suffer from the same disease for different genetic reasons until the responsible mutations are recognized. A chromosome region (locus) may cosegregate with a disease in some families but not in others reflecting heterogeneity of linkage. Alternatively different mutations at the same locus may be responsible.

3) Polygenic inheritance. Some traits may require the simultaneous presence

of mutations in multiple genes (See section 1.4.2.1). This complicates genetic mapping because no single locus is strictly required to produce a discrete trait.

4) Other transmission mechanisms. These include mitochondrial inheritance in which mitochondria pass solely through the maternal germ line and each meiotic transmission may involve selection from a potential mixed population of mutant and normal organelles; Genomic imprinting, which is a phenomenon whereby a nuclear gene or set of genes is differentially expressed according to whether or not it is of maternal or paternal origin; and the so-called "dynamic mutations" such as the expansion of trinucleotide repeats which have shown to be involved in the mechanisms of anticipation. These modes of transmission can complicate genetic analysis when they lead to highly variable transmission rates (Lander et al, 1994).

In addition, linkage analysis is feasible only if there are an adequate number of polymorphic genetic markers spaced evenly throughout the genome so that all possible loci of medical interest will be linked to some marker. In recent years, recombinant DNA technology has made it possible to construct a nearly complete linkage map of the human genome, which is continually being improved in both resolution and range with the addition of ever more markers. These new genetic markers and maps have been used in several genome scanning studies in schizophrenia (see section 1.5.3.3). However, linkage studies of schizophrenia based on candidate genes (section 1.5.3.2) can be limited by the availability of polymorphisms or genetic markers found within or at least nearby the gene(s) of interest. This is the reason why only two glutamate receptor subunit genes and a glutamate/aspartate transporter gene were able to be examined in this study. All the genetic markers used for the three loci studied for this research are localized at the 3'-untranslated region of the respective genes of interest and they are quite informative. Attempts are on going to identify polymorphisms in other glutamate receptor subunit gene loci in this laboratory, unfortunately no such polymorphisms have yet been found.

For a successful linkage strategy it is probably best to conceptualize schizophrenia as not one disorder but many with similar phenotypes. "The schizophrenias" may have more than one genetic and environmental cause. The genetic subtypes will be further subdivided by different modes of inheritance,

different epistatic genetic effects and environmental factors. It now seems that several loci have been identified in multiple family samples which increase susceptibility to schizophrenia. Large scale collaborative studies such as that by the European Science Foundation (ESF) network in the molecular neurobiology of mental illness have also confirmed several linkages. The genome scan on the 23 pedigrees used in this study is currently being carried out and will implicate or exclude other glutamate receptor loci in the genetics of schizophrenia.

On the other hand, linkage analysis in mice has identified two potential loci for non-obese diabetes (Todd et al, 1991). Unfortunately however, a mouse model for schizophrenia seems unlikely. A gene in the insulin-IGF2 region on human chromosome 11p has been identified for HLA-DR4-dependent diabetes susceptibility (Julier et al, 1991). Diabetes has genetic and environmental subtypes with a number of loci contributing to the risk, however potential susceptibility loci are being identified, for example close linkage between the candidate gene glucokinase on chromosome 7 to early onset non-insulin dependent diabetes mellitus (Froguel et al, 1992). The autosomal dominant form of retinitis pigmentosa is both clinically and genetically heterogeneous. Subdivided into two groups based primarily on age of onset, there is considerable within-family variation in expression, variable penetrance, and late age of onset cases. Nevertheless using single large multiplex families in three separate studies, three loci have been identified: chromosome 3, 6 (Farrar, 1990), and chromosome 8 (Blanton et al, 1991). A new subtype, X-linked, retinitis pigmentosa was also reported (McGuire et al, 1995). This demonstrates the advantage of using large enough pedigrees to show a significant lod on their own in mapping genetically heterogeneous diseases.

Alzheimer's disease is also genetically heterogeneous (St George-Hyslop, 1990; 1994) with some arbitrarily chosen early age of onset families (< 65 years) showing linkage to chromosome 21. The gene encoding amyloid precursor protein (APP), which is the precursor of the beta peptide of amyloid protein plaques and a strong candidate gene for Alzheimer's disease, was mapped to the same region of chromosome 21 (AD1 locus). This candidate was originally excluded by the demonstration of recombinants in a few families (Van Broeckhoven et al, 1987) but subsequent families which were compatible for linkage with APP identified rare

mutations causing the disease (St. George-Hyslop et al, 1990; Chartier-Harlin et al, 1991; Van Broeckhoven et al, 1992). This is an example to show that over zealous exclusion of strong candidate genes is not wise for a genetically heterogeneous diseases. Recently two other genes, named presenilin 1 (PS1 or S182 on chromosome 14, AD3 locus) and presenilin 2 (PS2 or STM2 E5-1 on chromosome 1, AD4 locus) respectively, and missense mutations for the early-onset type Alzheimer's disease with autosomal dominant pattern of inheritance and complete penetrance were also found by identification of the susceptible loci resulting from linkage studies (Sherrington et al, 1995; Levy-Lahad et al 1995; Rogaev et al, 1995). Although the exact mechanisms by which Alzheimer-type neurodegeneration occurs in the brains of humans heterozygous for these mutations are still yet to be defined, these observations have confirmed the prior hypothesis that there is more than one genetic defect contributing to this disease. This has given important implications for other areas of research into this distressing disorder.

However the cloning of such susceptibility loci for schizophrenia presents another set of obstacles resulting from the unique nature of this disease. Firstly, from the World Health Organization (WHO) ten-country study of incidence, Jablensky et al (1992) concluded: "...schizophrenic illnesses are ubiquitous, appear with similar incidence in different cultures and have clinical features that are more remarkable by their similarity across cultures than by their difference". It seems that in this respect schizophrenia differs from other common diseases such as coronary artery disease, diabetes, and arthritis which seem to vary more in different ethnic groups. Secondly, unlike Huntington's disease or Alzheimer's disease schizophrenia presents within the reproductive phase of life and is associated with a substantial biological disadvantage, greater in males than females (Vogel, 1979). Therefore, predisposing genes are exposed to a substantial selective pressure. Thirdly, several studies have generally agreed that enlargement of the cerebral ventricles applies to a subgroup of schizophrenics and not necessarily to all schizophrenics (section 1.6.5).

A few directions may be considered for the future research, in both genome scan and candidate gene approach: 1) Adequate definition of the phenotype. Attempts have been made to try to show that clinical heterogeneity of the illness

reflects a heterogeneity in the underlying neuropathology. However, attempts to divide the illness into discrete subtypes have been only partially successful. The classic subdivision into simple, catatonic, hebephrenic, and paranoid schizophrenia is unsatisfactory because many cases show features characteristic of more than one subtype. Liddle (1987) examined the pattern of correlation between schizophrenic symptoms in a group of patients with similar chronicity and found that symptoms segregated into three distinguishable syndromes: psychomotor poverty, disorganization, and reality distortion. It has been shown that each syndrome was associated with a specific pattern of cerebral blood flow perfusion (Liddle et al, 1992). Tsuang (1994) used the term "psychiatric genetic nosology" to refer to a scientific nosology created from psychiatric genetic data. A psychiatric genetic nosology seeks to classify patients into categories that correspond to distinct genetic entities. It may be that epidemiologic nosologies should be tailored to specific research questions. In addition, the concept of epidemiologic nosologies also recognizes that diagnostic criteria need not to be limited to the traditional signs and symptoms of psychiatric illness. For example, there have already been many sophisticated neurodiagnostic measures ranging from neuropsychological assessment to brain imaging techniques (section 1.6.3 and 1.6.5). However, these measures are usually considered dependent variables or outcomes rather than criteria for syndrome definition. It may be neurodiagnostic criteria, for example, that will carve out psychopathological subtypes which are more homogeneous with regard to the mechanisms of aetiology and pathophysiology than those defined by traditional approaches.

2) More sophisticated genetic models. Unlike familial Alzheimer's disease and Huntington's disease, schizophrenia does not show a clear mode of transmission (Faraone et al, 1985). If schizophrenia is a developmental disorder (section 2.3.3), epistasis is very likely to exist. It could be considered as a two-locus system in which neither locus has a major effect on its own (Frankel et al, 1996; Kidd, 1997). Two-locus linkage analyses may need to consider segregation at all possible pairs of genome regions and thus require complete coverage of the genome with highly informative markers. Also, different models of epistasis need to be considered for each pair of regions. Since the marginal effects of one locus are dependent on the

allele frequencies at the interacting locus or loci, one needs to think of population variation (Kidd, 1997). It has been shown that expressed polymorphisms consisting of normal alleles with functional differences have quite large frequency differences among populations. Examples for genes of neuropsychiatric relevance include the genes for dopamine DRD4 (Chang et al, 1996), dopamine β-hydroxylase (DBH, Kidd, 1997), and ciliary neurotrophic factor (CNTF, Gelernter et al, 1997). If any of these loci with functionally variant alleles are part of an epistatic system underlying susceptibility to schizophrenia, the allele frequency variation would be a systemic factor across populations.

3) Quantitative trait loci (QTL) approach. A general principle of statistics is that analysis is more powerful when continuous rather than categorical variables are employed. Genes influencing scores on continuous measures are known as quantitative trait loci (QTL). Both linkage and association methods have been developed to map QTLs in humans and successes have been achieved for reading disability (Plomin et al, 1994; Cardon, 1994). Psychiatric genetics has traditionally focused on categorical phenotypes. There has been evidence that common disorders represent the quantitative extremes of continuous dimensions (Plomin et al, 1994). Therefore, a QTL approach may be feasible if valid continuous measures of traits related to schizophrenia can be developed. Risch and Zhang (1995) have pointed out that the optimal design for a QTL sib-pair linkage analysis is to use a pair of sibs which are extremely discordant for the variable. This approach is a more powerful design in contrast to the situation of a dichotomous variable, e.g., affected and unaffected.

6.2 Mechanisms underlying the regulation of glutamate receptor subunit gene expression in response to antipsychotic drugs and their clinical correlates

Although the direct genetic evidence of the contribution of the allelic variation at glutamate receptor subunit gene loci to schizophrenia susceptibility has not been found yet, the gene expression studies did demonstrate that the glutamate receptor

subunits, especially the NMDA subtype receptors, are involved in the molecular mechanisms of the action of the widely used antipsychotic drug flupenthixol.

A major focus of current antipsychotic drug research is the identification of the chronic adaptations these drugs induce in the brain that underlie their antipsychotic effects and some of their long-term side effects, such as tardive dyskinesia. Two general approaches have been applied: electrophysiological and molecular. Through the electrophysiological approach, studies by Bunney and colleagues (reviews by Grace et al., 1997) have shown that repeated antipsychotic drug treatment results in a delayed inactivation of dopamine-neuron firing in the midbrain due to depolarization block which correlates with the therapeutic efficacy of antipsychotic drugs in human. However, how depolarization block could have an impact on psychotic symptoms remains unknown. The molecular approach has been to focus on the regulation of gene expression by antipsychotic drugs. With prolonged exposure to the drugs, it is hypothesized that persistent antagonism of dopamine receptors lead to changes in gene expression and hence in functional properties of the dopaminergic and dopaminoceptive neurons. Changes in gene expression and function might also occur in neurons innervated by dopaminoceptive neurons, in neurons innervated by those cells, and so on (Hyman et al, 1996). However, the particular neurons or genes in which the critical changes responsible for antipsychotic drug efficacy occur remain unknown.

Most studies were carried out with the traditional typical antipsychotic drug haloperidol or the atypical antipsychotic drug clozapine. Using in situ hybridization technique, Meshul et al (1996) found that subchronic (28 days) administration of haloperidol or clozapine significantly decreased the abundance of NMDAR1 subunit mRNA containing a 63-base insert in the rat caudate nucleus. Both drugs increased the mRNA levels for NMDAR2B, however, only the increase caused by clozapine reached a statistically significant level. In addition, both drugs had no effect on ^{3}H-MK801 binding to striatal membrane preparations. These results are consistent with our findings. Clozapine and haloperidol both displace the binding of ^{3}H-MK801 from several different tissue preparations (Lindsky et al, 1993) and furthermore haloperidol may interact with the glycine binding site on the NMDA receptor ion channel complex (Fletcher et al, 1993). A recent report by Wang et al (1996)

indicated that the NMDAR1 splice variants containing a 21-amino acid insert may be located presynaptically. It appears that antipsychotic drugs may cause a selective decrease in NMDAR1 mRNA for presynaptic receptors. The change in levels of mRNA for NMDA receptor subunits could be dependent on the extent of antipsychotic drug-induced alteration of glutamate release. Two reports (Yamamoto et al 1994; See et al, 1994) using in vivo microdialysis found a haloperidol-induced increase in extracellular glutamate in the striatum. This antipsychotic drug-induced increase in synaptic glutamate activity is also associated with an increase in the density of asymmetric synapses containing a perforated postsynaptic density (PSD, Meshul et al, 1996).

In addition to the direct action on the NMDA receptor channel complex, antipsychotic drugs could also regulate the gene expression of the glutamate receptor subunits via indirect mechanisms, i.e., through interaction with other neurotransmitter systems (section 3.2.1) or through postreceptor events and intracellular pathways. The interaction between glutamatergic and dopaminergic neurons in the brain was summarized in Section 2.3.2. Glutamatergic pyramidal neurons that project to subcortical regions, e.g. striatum and nucleus accumbens, are regulated by most major neurotransmitters including dopamine. Cortical dopaminergic neurotransmission inhibits these pyramidal neurons directly or indirectly by increasing GABA release from interneurons (Deutch, 1993; Gellman et al, 1994). The removal of cortical dopamine, therefore, would be expected to increase dopamine release and metabolism in the striatum and nucleus accumbens by increasing the activity of glutamatergic pyramidal neurons which project to the vicinity of dopaminergic nerve terminals in these regions. Evidence for reductions in the number of GABAergic interneurons (Benes et al, 1995) and compensatory increase in $GABA_A$ receptors (Benes et al, 1992) in the prefrontal cortex of schizophrenic patients further supports this concept of corticofugal disinhibition. Moreover, evidence in rodents that haloperidol increases GABA immunoreactivity in synaptic terminal with prefrontal cortical pyramidal neurons was also reported (Vincent et al., 1994). With regard to the intracellular pathway, dopamine is known to affect intracellular messengers such as the cAMP response element binding (CREB) protein, c-fos, and cAMP. The regulation of NMDA receptor genes by

antipsychotic drugs may be a result of their influence of such messengers because the promoter of the NMDAR1 gene has CRE, AP-1, and AP-2 regulatory elements (Bai et al, 1993).

We chose to study flupenthixol as an initial investigation of antipsychotic drug regulation of glutamate receptor subunits because of the scientific advantage gained from the diverse effects of its cis- and trans- geometric isomers. The previous clinical trial (Johnstone et al., 1978) of cis (α) flupenthixol showed a significant superiority over its trans (ß) isomer in the amelioration of positive symptoms --- delusions, hallucinations, and incoherence of speech (thought disorder). The differences between the treatments with these two isomers were found to be negligible for negative symptoms (flattening of affect and muteness) and for non-specific symptoms (depression, anxiety, and retardation). A previous study in this laboratory (De La Chocha et al, 1991) demonstrated a two fold increase in the abundance of dopamine D_2 receptor mRNA in the half mouse brain after ten week treatment with cis- but not trans- flupenthixol.

The finding in the present study that both trans- and cis- isomers of flupenthixol regulated the mRNA levels of certain NMDA receptor subunits to a similar degree suggests that the NMDA receptors may be involved in the pathophysiology of schizophrenic symptoms other than positive symptoms. This hypothesis is also supported by a few clinical studies in which improvement of negative symptoms in chronic schizophrenic patients by glycine therapy was reported (section 2.3.6). The fact that D_2 mRNA level was more affected by cis- than trans-flupenthixol while both isomers affected that of NMDA receptor subunits to a similar extent also implies that flupenthixol might regulate NMDA receptor gene expression through mechanisms independently of the D_2 receptor. Cis-flupenthixol has a 30 and 20 fold higher affinity to D_1 and D_2 receptors respectively than its trans-isomer (Faedda et al., 1989). The D_1 : D_2 potency ratio for cis-flupenthixol is 1.58 and that for trans-flupenthixol is 1.08. However, there is no difference between the affinities of these two isomers at opiate, muscarinic, and GABA receptor sites nor in their ability to inhibit GABA uptake into brain synaptosomes (Enna et al., 1976). All of these above-mentioned neurotransmitters have been suggested as sites for the action of antipsychotic drugs as well as for the increase in susceptibility to schizophrenia

(Benes, 1995; Hyman et al., 1996; Kinon et al., 1996).

Further work is needed to define the mRNA change in a finer region in the brain, such as hippocampus, striatum, nucleus accumbens, amygdala and so on, which are areas of interest for the action of antipsychotic drugs. At present this work must be done by means of in situ hybridization which is much less sensitive in terms of mRNA quantification than northern blotting or the solution hybridization technique we have used. Change of NMDA receptor subunit gene expression at the protein level detected by immunoblotting with available antibodies is not as obvious as initially expected from the data of mRNA levels. This could be due to the sensitivity of western blotting which is known as very specific yet less sensitive in quantification. In addition, it has become apparent that changes in mRNA and protein stability probably contribute to neurotransmitter-induced changes in protein levels (Hyman et al., 1993). The stability and translatability of specific mRNAs have been shown to be regulated by cyclic AMP, Ca^{+2}-dependent mechanisms, and a family of proteases have been shown to be specifically activated by increases in cellular Ca^{+2} levels. The multitude of mechanisms by which amounts of proteins can be regulated despite a constant rate of mRNA translation in transcriptional, post-transcriptional, and post-translational levels underscores the complex mechanisms utilized by neurons to maintain their homeostatic.

It would be difficult to make inferences about the pathophysiology of schizophrenia or any other psychiatric disorders based on drug mechanisms. The complexity of the inter- and intracellular connections with their multiplicity of interconnected control systems and feedback loops implies that therapeutically effective drugs may be acting on the brain in several steps or levels removed from any pathophysiological lesions. For example, the dopamine antagonism of most effective antipsychotic drugs can not, however, lead to the conclusion that schizophrenia is caused by a primary problem in dopamine neurons or dopamine receptors. This concern is particularly serious for psychiatric disorders that are very likely aetiologically heterogeneous where the fundamental pathophysiological problem may differ across patient groups with only downstream neuronal consequences in common. The history in search of the genes for Alzheimer's disease (section 6.1) has given a good example. In addition, the fact that

neuroanatomic circuits between neurons as well as biochemical circuits within neurons must process and integrate complex information reveals an extraordinary degree of independence of signaling systems at both inter- and intra- cellular levels. This implies that a perturbation in one system will influence many others. Moreover, chronic perturbations in a system may result in homeostatic adaptations in numerous other systems to compensate for the original perturbation. Therefore, a drug that produces its immediate action on a certain signal transduction pathway in a particular group of neuron will produce short- and long-term alterations in many other pathways in the same or other groups of neuron. However, understanding these downstream and often delayed effects of antipsychotic drugs and by analogy the effects of other perturbations of the brain would be one of the most feasible ways of achieving a molecular understanding of schizophrenia.

In summary, the gene expression of specific NMDA receptor subunits in several regions of the left rat brain was regulated by the treatment with either cis- or trans-isomer of the antipsychotic drug flupenthixol. The NR1 mRNA was generally decreased throughout the 24 weeks treatment with trans- flupenthixol and after long-term (12 or 24 week) treatment with cis- flupenthixol in the frontal and subcortical areas. NR2B and NR2C mRNA expression demonstrated a dynamic pattern of change in different brain regions following treatment with flupenthixol whilst NR2A and NR2D gene expression was relatively unaffected except in the subcortical region. The gene expression of AMPA and kainate types of glutamate receptor subunits was unaffected following 4 and 24 week treatment with either trans- or cis-flupenthixol. These results, nevertheless, indicate that adaptations in glutamate receptors may represent an important and novel mechanism through which neuroleptics exert some of their effects on brain function.

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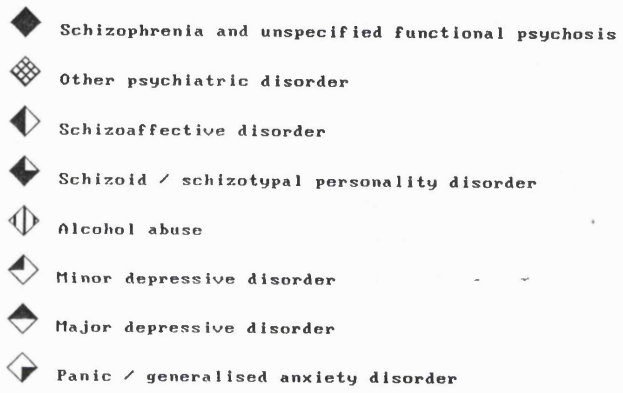
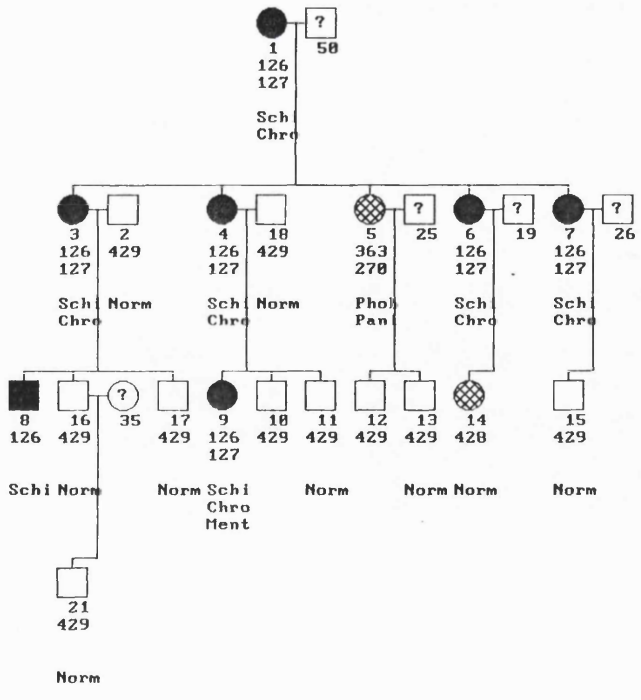
APPENDIX 1 Pedigree structures and diagnoses of the 23 English and Icelandic schizophrenic families





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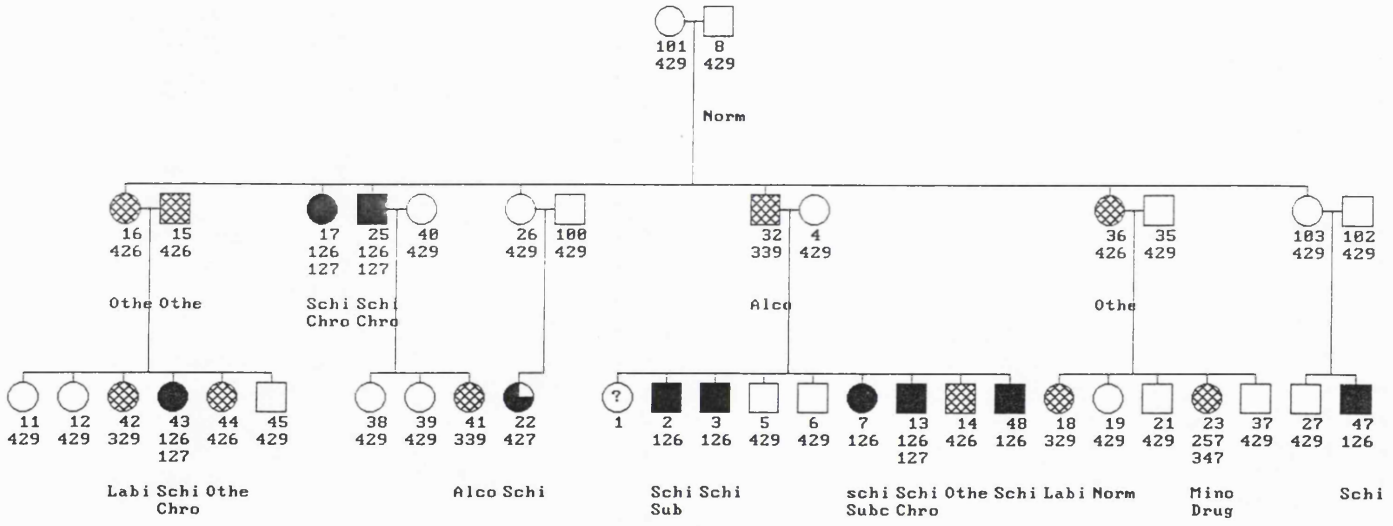
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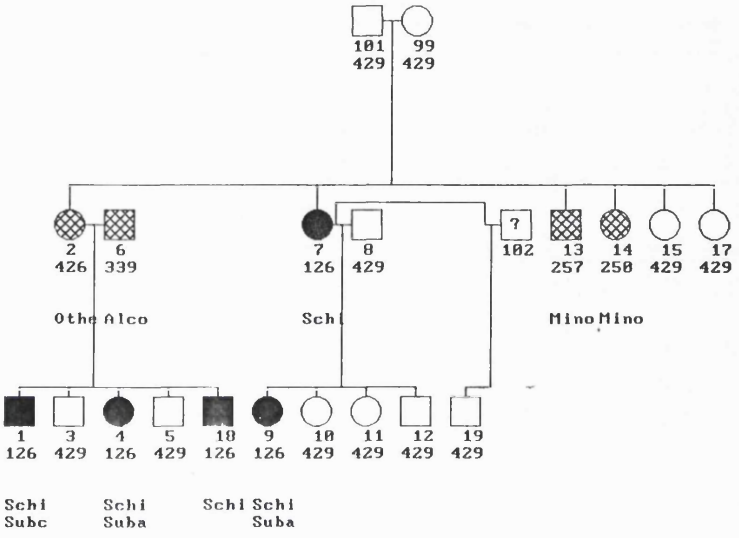
Pedigree 27



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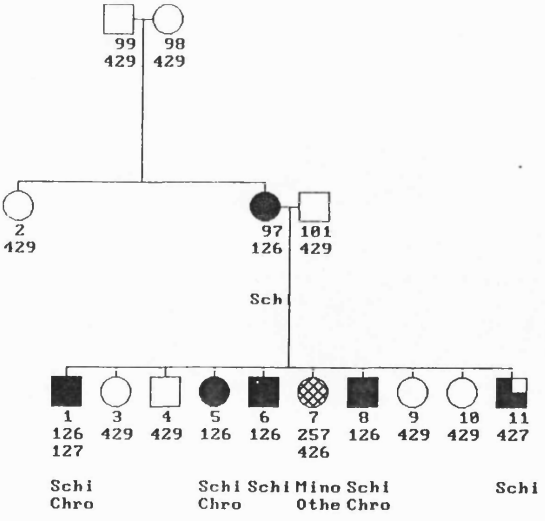

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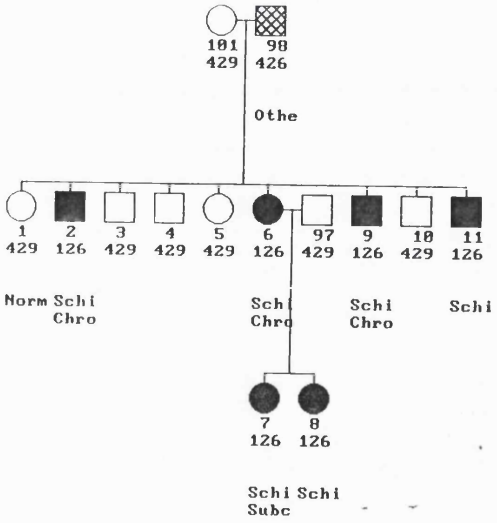

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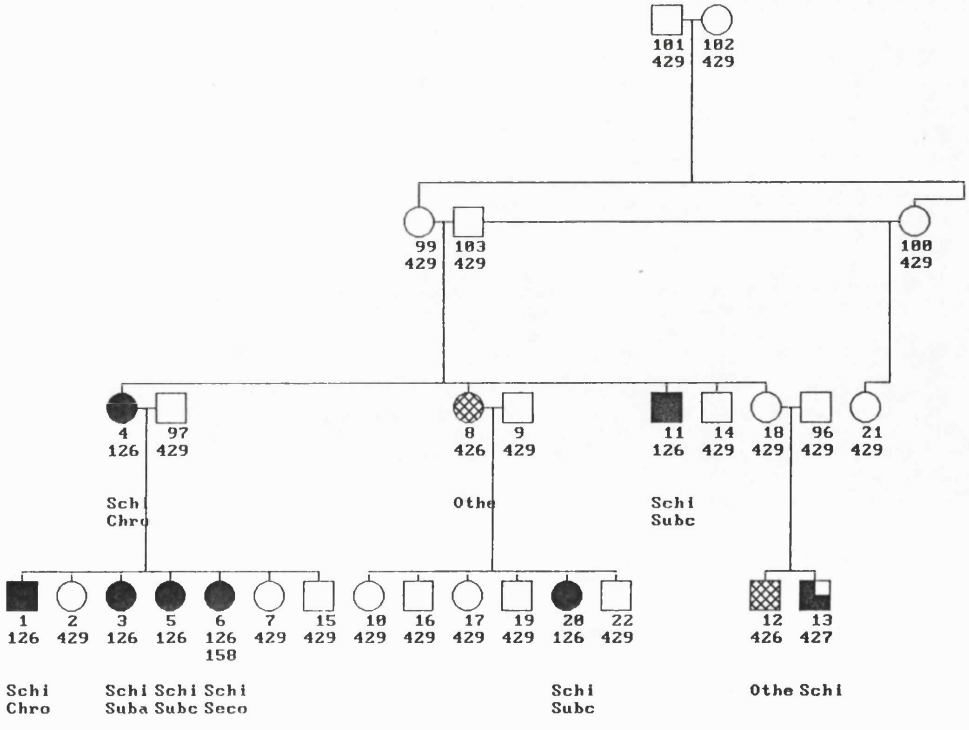

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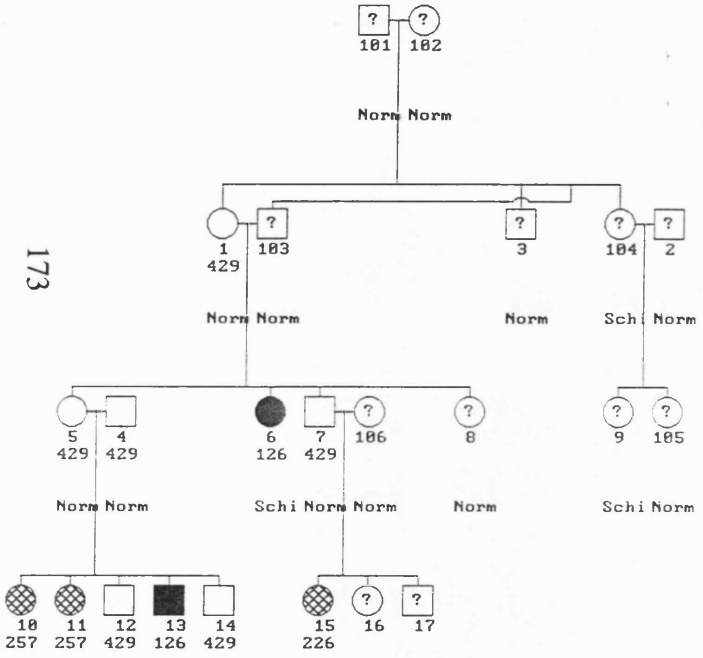

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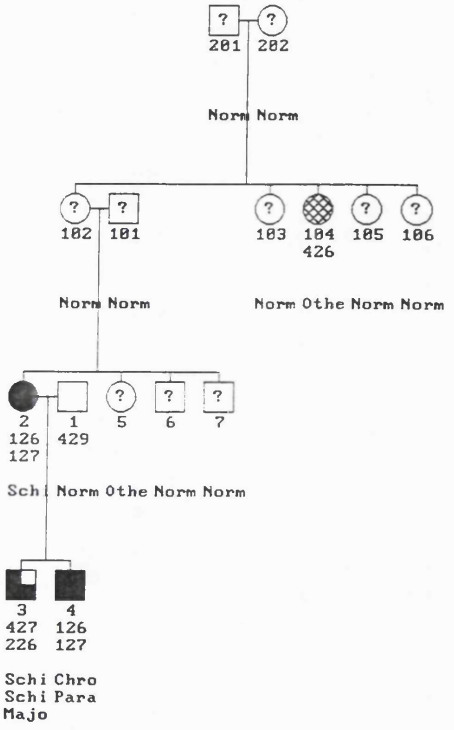

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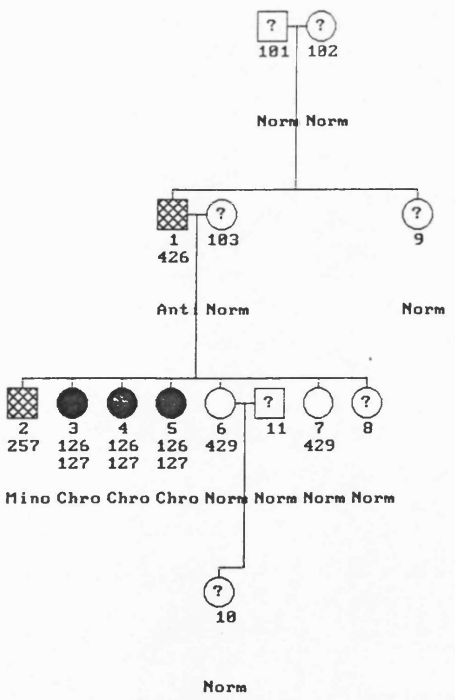

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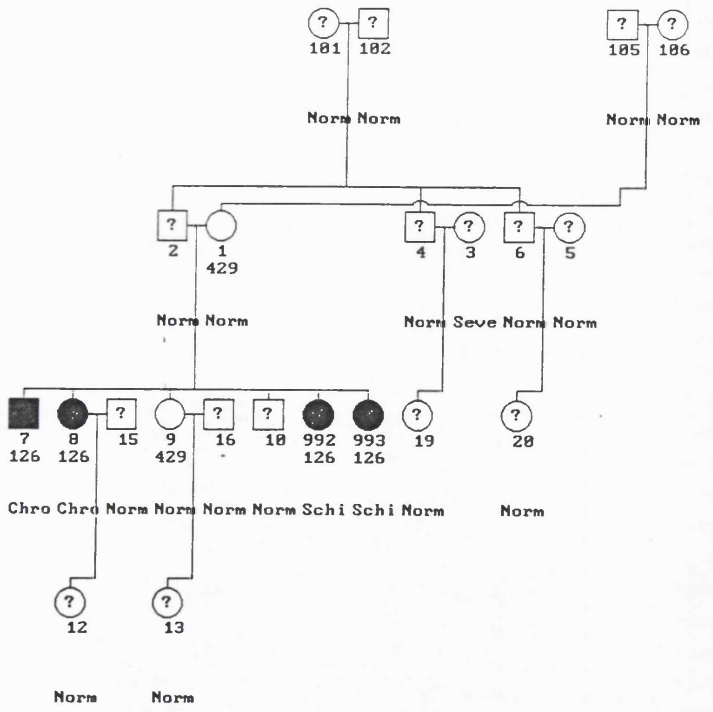

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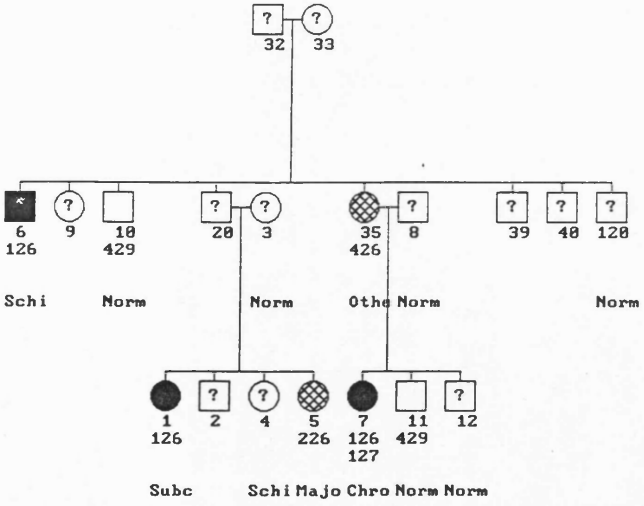

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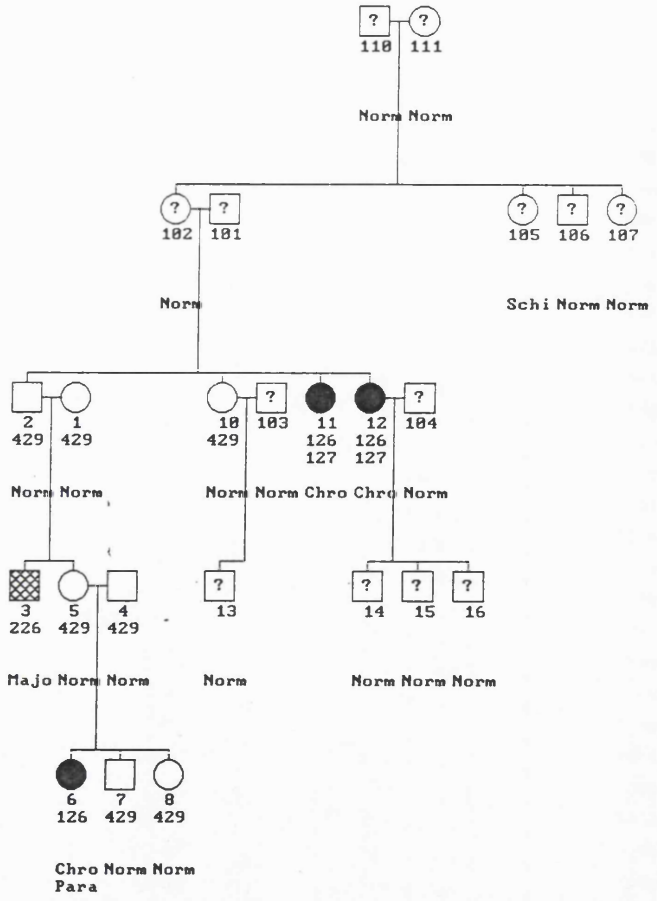

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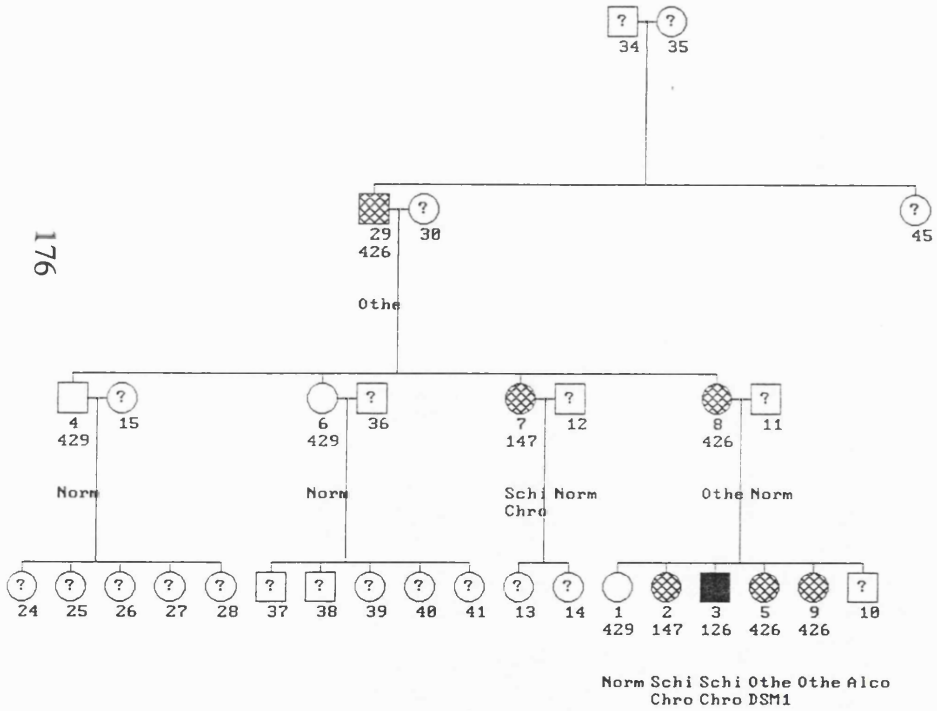

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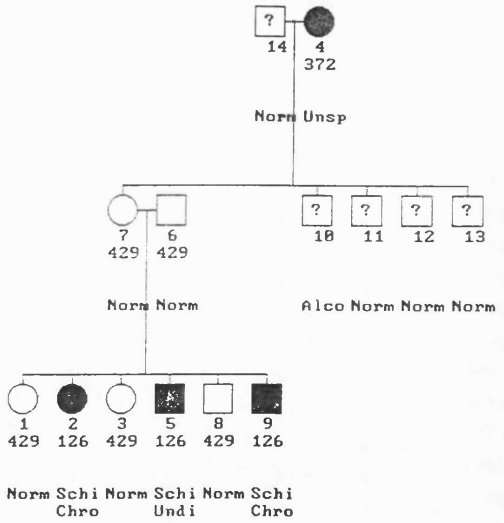

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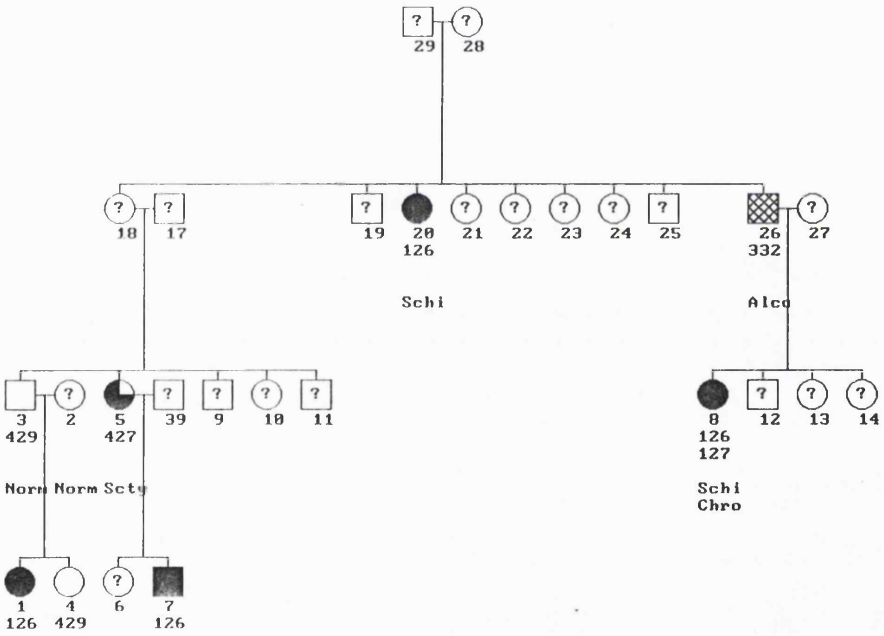

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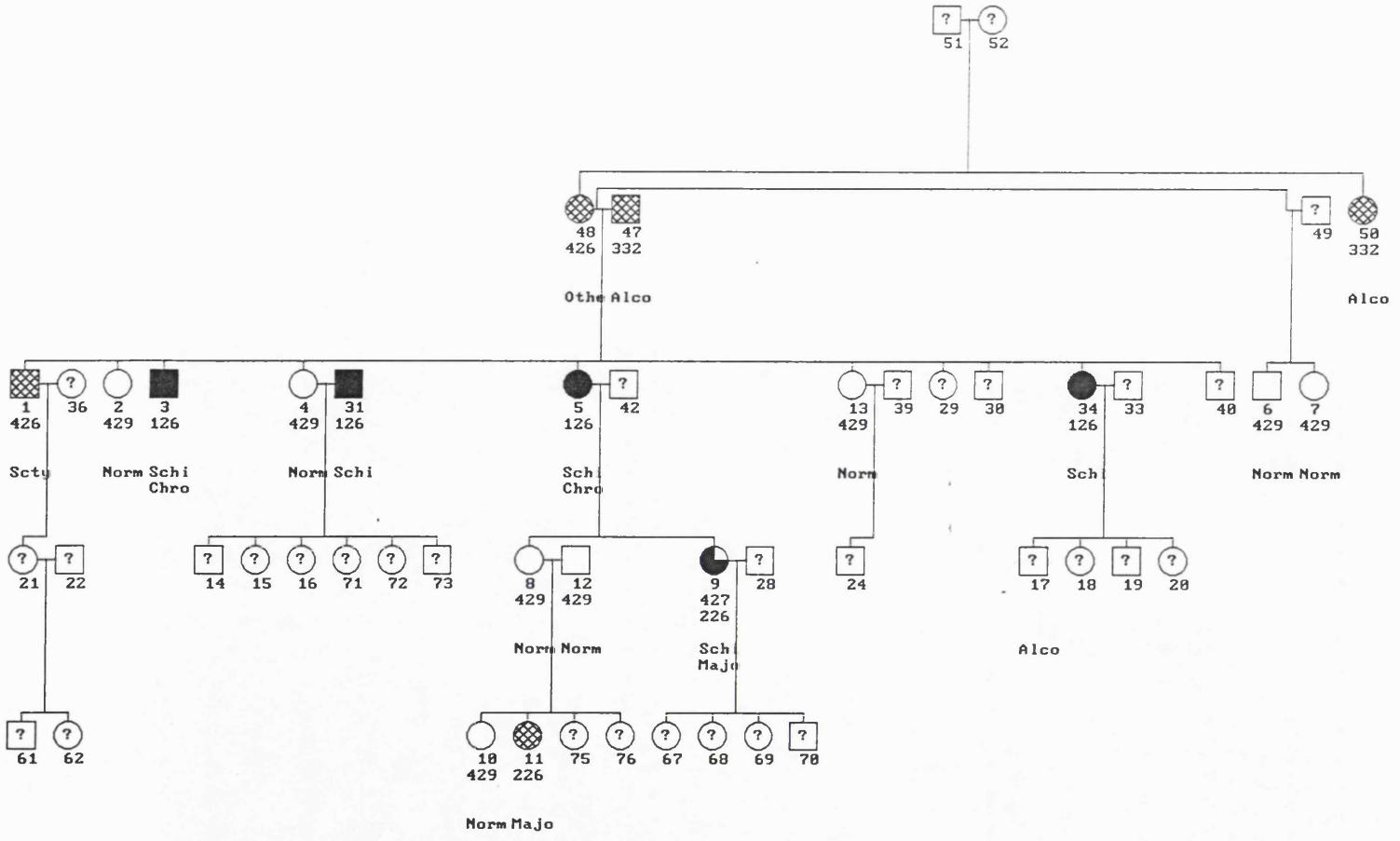

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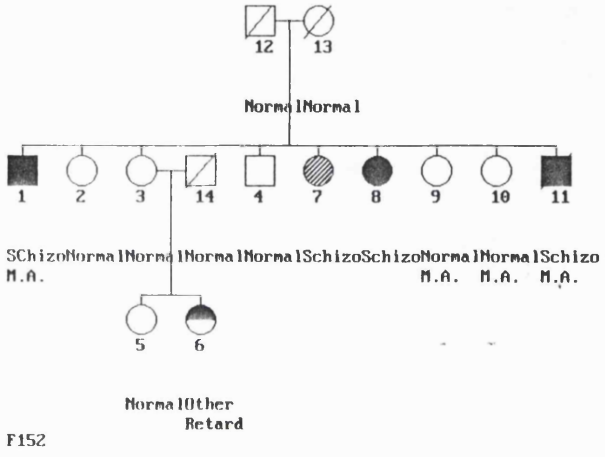

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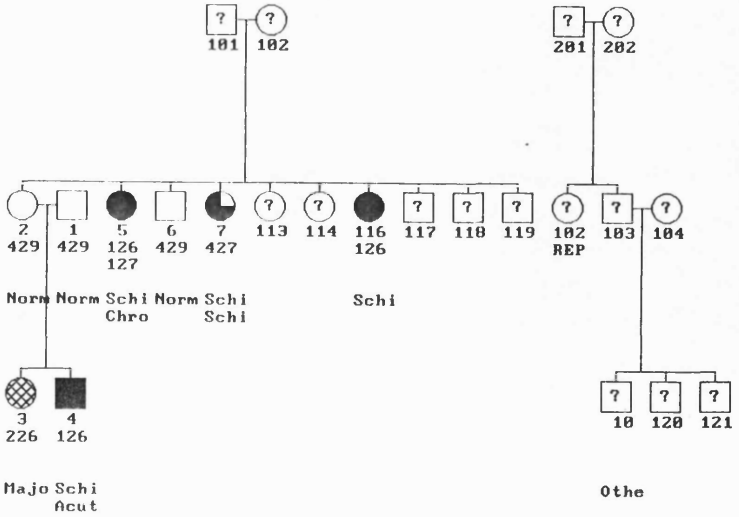
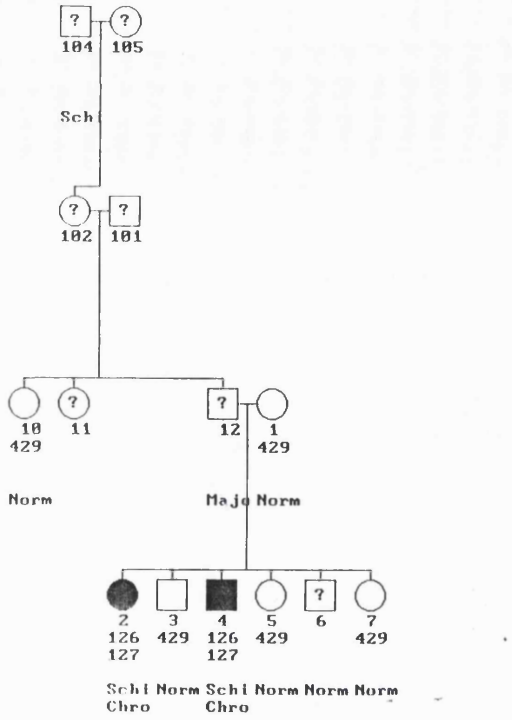


Pedigree 153



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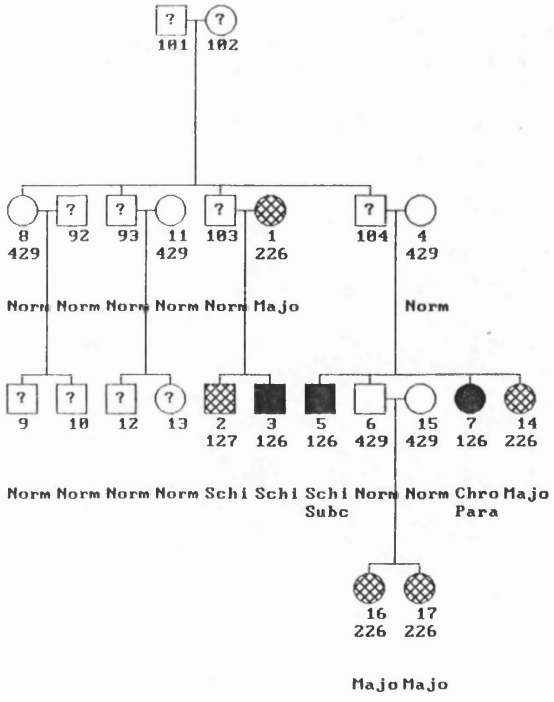

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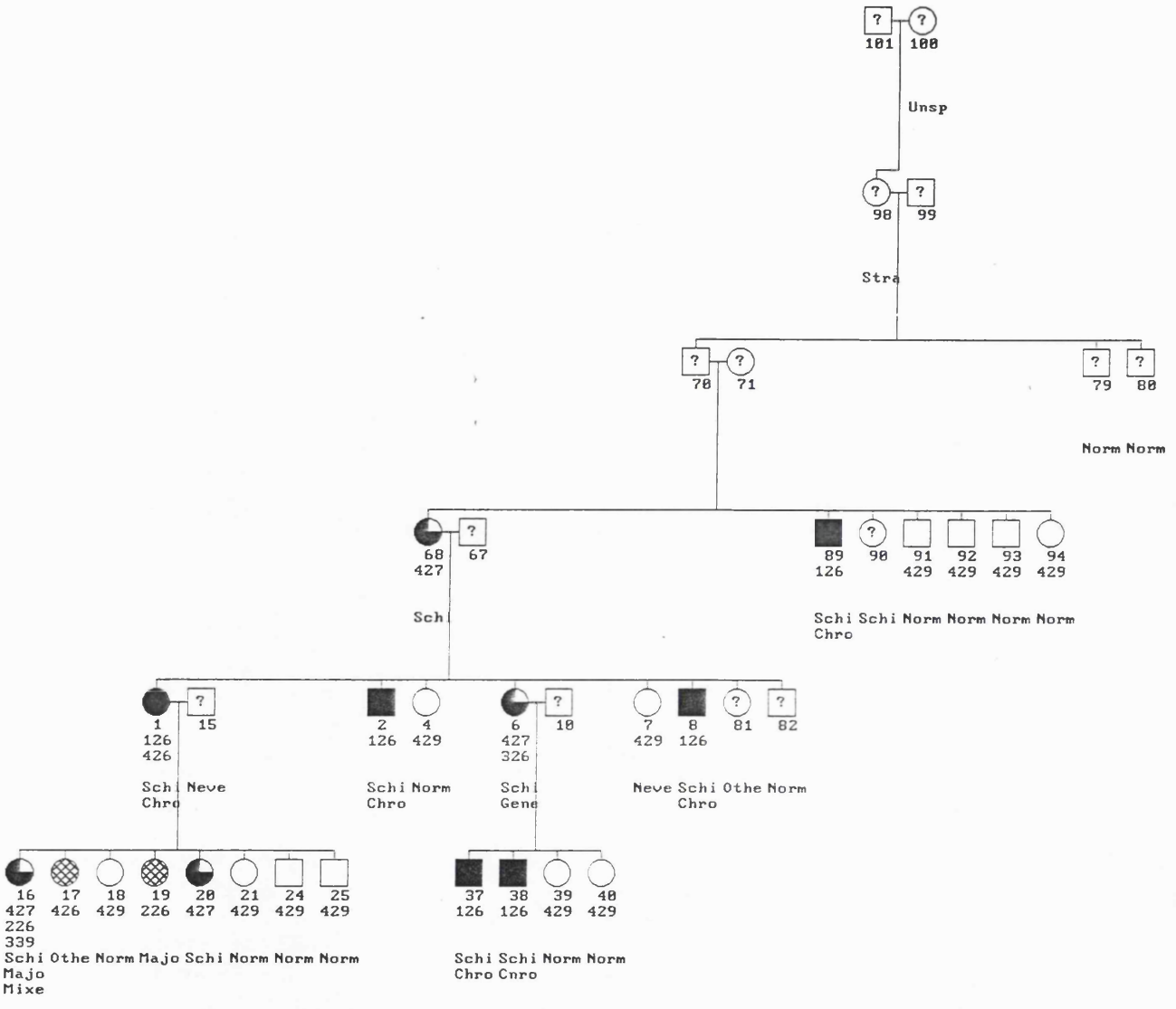

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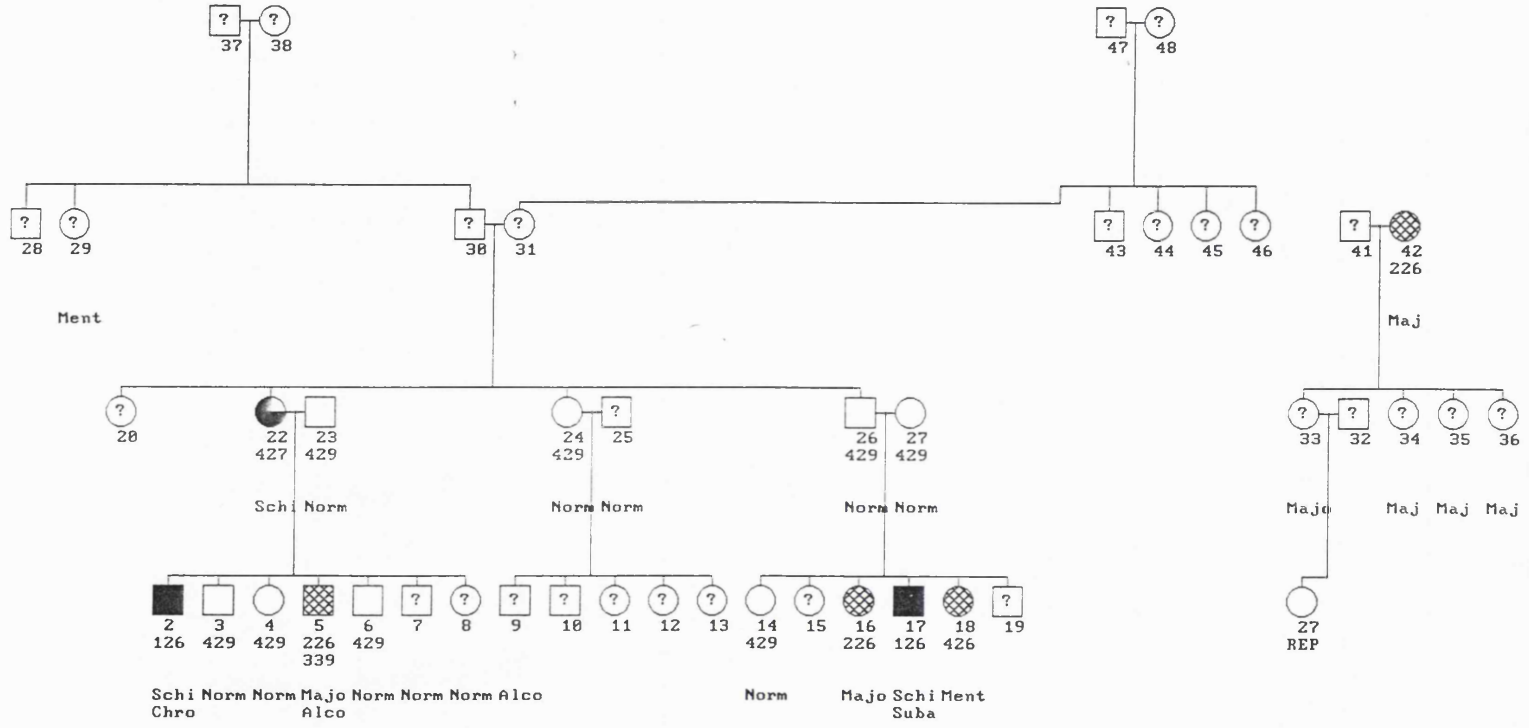

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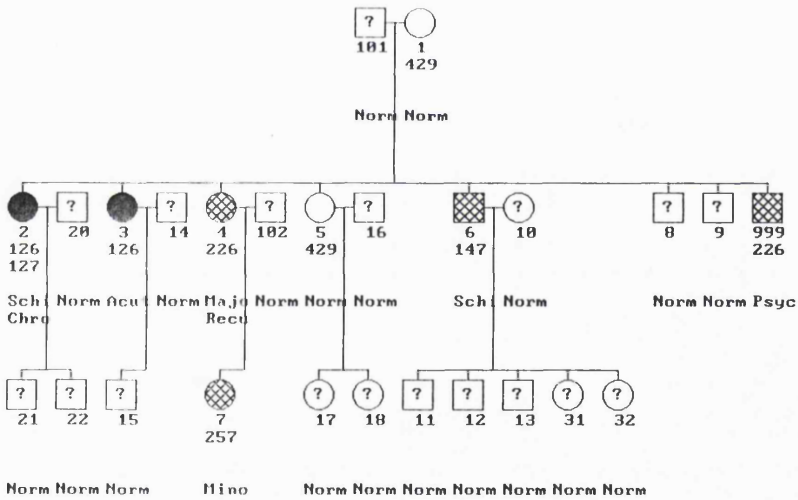

Pedigree 250

APPENDIX 2 Relevant publications resulting from this thesis

Chen ACH, Kalsi G, Brynjolfsson J, Sigmundsson T, Curtis D, Butler R, Read T, Murphy P, Petursson H, Barnard EA, and Gurling HMD. (1996) Lack of evidence for close linkage of the glutamate GluR6 receptor gene with schizophrenia. Am J Psychiat 153:1634-1636

Chen ACH, Kalsi G, Brynjolfsson J, Sigmundsson T, Curtis D, Butler R, Read T, Murphy P, Barnard EA, Petursson H, and Gurling HMD. (1997) Exclusion of linkage between schizophrenia and the gene encoding a neutral amino acid glutamate/aspartate transporter, SLC1A5. Am J Med Genet 74:50-52

Chen ACH, Kalsi G, Brynjolfsson J, Sigmundsson T, Curtis D, Butler R, Read T, Murphy P, Petursson H, Barnard EA, and Gurling HMD. (1997) Exclusion of linkage of schizophrenia to the gene for the glutamate GluR5 receptor. Biol Psychiatry 41:243-245

Chen ACH, McDonald B, Moss SJ, Gurling HMD. (1997) Gene expression studies of mRNAs encoding the NMDA receptor subunit NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C, and NMDAR2D following long term treatment with cis and trans flupenthixol as a model for understanding the mode of action of schizophrenia drug treatments. Mol Brain Res (in press)

Chen ACH, and Gurling HMD. Lack of effect of trans- and cis-flupenthixol on AMPA and kainate glutamate receptor gene expression in rat brains. (Manuscript in preparation)

ABSTRACTS & PRESENTATIONS:

* Biomedicine '97 (Joint meeting of Association of American physicians, American society for clinical investigation, and American federation for medical research). Washington, DC, U.S.A., April, 1997
Chen ACH**, Gurling HMD. (1997) Regulation of NMDA receptor subunit gene expression by both cis- and trans- flupenthixol. J Invest Med (in press)
** Trainee Investigator Awards

* The international society of psychiatric genetics symposium. San Francisco, U.S.A., October, 1996
Chen ACH, Kalsi G, Brynjolfsson J, Sigmundsson T, Curtis D, Butler R, Read T, Murphy P, Barnard EA, Petursson H, and Gurling HMD. (1996) Exclusion of linkage between schizophrenia and the gene encoding a neutral amino acid glutamate/aspartate transporter, SLC1A5. Psychiatric Genetics 6:138

* The 4th world congress on psychiatric genetics. Cardiff, U.K., August, 1995
Chen ACH, Kalsi G, Brynjolfsson J, Sigmundsson T, Butler R, Sharma T, Read T, Murphy P, Petursson H, Gurling H. (1995) Genetic linkage analysis of the GluR5 and GluR6 glutamate neuroreceptor genes in schizophrenia. Psychiatric Genetics 5: S57