https://theses.gla.ac.uk/

Theses Digitisation:
https://www.gla.ac.uk/mygla/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge
This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given
TRANSCRIPTOME ANALYSIS OF LASER-MICRODISSECTED RAT BRAIN REGIONS RELEVANT TO COGNITION AND PSYCHIATRIC DISORDERS.

Thesis submitted by

Antonio Ferra

For the degree of
Doctor of Philosophy
of the University of Glasgow

March 2007
Faculty of Biomedical and Life Science
University of Glasgow
The copyright of this thesis belongs to the author under the terms of the United Kingdom Copyright Act as qualified by University of Glasgow regulation. Due acknowledgement must always be made of the use of any material contained in, or derived from, this thesis.
Acknowledgements:

I would like to thank my main supervisor, Dr. Susan Coelran, not only for her invaluable scientific advice, but also for her enthusiasm and friendly support.

I would also like to thank my supervisors Prof. Brian Morris and Prof. Judy Pratt for their help in the experimental design and constant advice.

Many thanks to all the staff and students at the Yoshitomi Research Institute for Neuroscience in Glasgow, for their support and for contributing to an enjoyable and peaceful work environment.

Lastly, I would like to thank Mitsubishi Pharma Corporation, Japan, for the financial support that made this work possible.
ABSTRACT
Schizophrenia is a severe psychiatric disorder associated with hallucinations, delusions, affective flattening, lack of motivation, social isolation and cognitive impairments. Features of the cognitive deficits observed in schizophrenic patients and in animal models of schizophrenia, such as attentional processes, cognition, working memory and behavioural flexibility, require the activation of the prefrontal cortex (PrL) and the ventral orbital cortex (VO), which are subregions of the prefrontal cortex. The thalamic reticular nucleus (Rt) also plays a key role in the regulation of these cortical functions by exerting an inhibitory control over the thalamo-cortical projections. The physiological role of a specific brain region could be associated with its gene expression profile. Moreover, the genes specifically expressed or enriched in one region could play a key role in the neuropathology of neuropsychiatric disorders.

The aim of the project was two-fold: to identify genes enriched in three brain regions implicated in some aspects of cognition and neurobiological alteration observed in schizophrenia models (PrL, VO and Rt) compared to one control region (primary motor cortex, M1); and to analyse the expression of selected enriched genes in a chronic PCP animal model of schizophrenia (Cochran et al. 2003).

The expression profiling of the PrL, VO and Rt was performed using a combination of laser-assisted microdissection, linear amplification and GeneChip microarray analysis. The microdissections were performed on coronal sections dissected from 6 naïve rat brains using the Leica AS LMD system. After extraction, the total RNA was linearly amplified, labelled and hybridised onto Affymetrix Rat Genome 230 2.0 arrays. The data were analysed according to the Rank Product method (Breitling et al., 2004). The regional overexpression of some selected genes was validated by in situ hybridisation (ISH) on six naïve brains. The expression of these genes was then analysed by ISH after a chronic intermittent PCP treatment (2.58mg/kg) in six treated and six control rat brains.
This study shows that the expression of many genes is differentially regulated in the selected areas. In the Rt, 429 transcripts were overexpressed compared to the cortical areas. Intra-cortical variations were also observed: 233 transcripts were overexpressed in the Prl compared to the other cortical areas, whereas 83 transcripts were enriched in the VO. The selectively expressed genes were involved in several biological mechanisms, such as neurotransmission, ion transport, neuronal development and synaptic plasticity. Ten genes were selected according to their levels of expression for the validation of both small and large differential expression by ISH. Expression analysis by ISH confirmed the overexpression in the PrL, VO and Rt predicted by the LMD-microarray for all genes tested. Wolframin, a gene which is likely to be involved in intracellular calcium trafficking that is the causative factor of Wolfram syndrome, showed significant decreased expression in the PrL in the PCP model of schizophrenia (p<0.05). Wolframin could be directly involved in the psychiatric conditions observed in the majority of Wolfram syndrome patients.

Genome-wide expression analysis combined with laser-assisted microdissection showed that anatomical and functional differences between brain regions are associated with differential gene expression. Alterations of the region-specific expression could be crucial for psychiatric diseases; therefore this approach is exceptionally useful for the selection of candidate genes to study in models of psychiatric disorders such as schizophrenia. Using the LMD-microarray approach followed by ISH validation, this project defined the gene expression profiles of the PrL, VO and Rt in the rat brain and identified a novel gene with decreased expression in a chronic PCP model of schizophrenia.
TABLE OF CONTENTS
1. INTRODUCTION

1.1 Schizophrenia

1.1.1 Diagnostic criteria for schizophrenia

1.1.2 Positive symptomatology of schizophrenia

1.1.3 Negative symptomatology of schizophrenia

1.1.4 Cognitive dysfunction in schizophrenia

1.1.5 Subtypes of schizophrenia

1.1.6 Pharmacotherapy of schizophrenia

1.2 The neuropathology of schizophrenia

1.2.1 Macroscopic alterations in the schizophrenic brain

1.2.2 Cytoarchitectural abnormalities in schizophrenia

1.3 Neurochemical abnormalities in schizophrenia

1.3.1 Dopamine hypothesis

1.3.2 Serotonergic system involvement

1.3.3 Glutamatergic hypothesis

1.3.4 GABAergic neurons: their interaction with dopaminergic and glutamatergic systems and role in schizophrenia.

1.4 Animal models of schizophrenia

1.4.1 Modelling prefrontal cortex dysfunction in rats: limitations and advantages

1.4.2 Environmental animal models of schizophrenia

1.4.3 Lesion models of schizophrenia

1.4.4 Pharmacological models of schizophrenia

1.4.5 Genetic models of schizophrenia

1.5 Schizophrenia and genes

1.5.1 Is schizophrenia correlated with specific gene alterations?

1.5.2 Microarray analysis of gene expression in schizophrenia
2.1 Materials

2.2 Laser-assisted microdissection/microarray study

2.2.1 Animals and tissue preparation
2.2.2 Histological staining
2.2.3 Immunohistochemical staining
2.2.4 Identification of regions and Laser Microdissection
2.2.5 Total RNA isolation
2.2.6 RNA amplification
2.2.7 Array target preparation and purification
2.2.8 Labelled aRNA fragmentation
2.2.9 Microarray Hybridisation
2.2.10 Microarray washing, staining and scanning
2.2.11 Normalisation and analysis

2.3 Confirmation of regional expression by in situ hybridisation

2.3.1 Probe design
2.3.2 Oligonucleotide labelling
2.3.3 Slide preparation and fixation
2.3.4 Hybridisation and negative controls
2.3.5 Statistical analysis

2.4 Expression of localised genes in a chronic PCP model of schizophrenia

2.4.1 Chronic PCP administration (chronic-intermittent PCP model of schizophrenia) and tissue preparation
2.4.2 In situ hybridisation
2.4.3 Statistical analysis
3. mRNA EXPRESSION PROFILING IN LASER-MICRODISSECTED BRAIN REGIONS

3.1 Introduction

3.1.1 Anatomical and functional heterogeneity of schizophrenia-related prefrontal cortical regions in the rat

3.1.2 The thalamic reticular nucleus: modulation of the PFC-dependent cognitive processes

3.1.3 From functional specificity to molecular characterisation: the importance of gene expression profiling in brain

3.1.4 Laser-assisted microdissection, linear amplification and microarray analysis: innovative tools for high-throughput expression analysis

3.1.5 Gene expression profiling via LMD/microarray in neuroscience research

3.2 Aims

3.3 Results

3.3.1 Tissue staining and microdissection

3.3.2 Total RNA quality control

3.3.3 Amplified aRNA quality control

3.3.4 Microarray hybridisation

3.3.5 Regional differential gene expression

3.3.6 Biological function of localised genes

3.4 Discussion

3.4.1 Functional classes of localised genes

3.4.2 Overexpression of neurodevelopment-related genes in the PrL

3.4.3 Localised expression of genes involved in neurotransmission

3.4.4 Localised expression of genes involved in ion transport
### 4. Confirmation of Regional Expression Profiles Generated by LMD/Microarray Using In Situ Hybridisation

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>117</td>
</tr>
<tr>
<td>4.1.1 Confirmation of microarray results</td>
<td>117</td>
</tr>
<tr>
<td>4.1.2 Genes overexpressed in the PrL</td>
<td>120</td>
</tr>
<tr>
<td>4.1.3 Genes overexpressed in the VO</td>
<td>126</td>
</tr>
<tr>
<td>4.1.4 Genes overexpressed in the Rt</td>
<td>128</td>
</tr>
<tr>
<td>4.2 Aims</td>
<td>137</td>
</tr>
<tr>
<td>4.3 Results</td>
<td>138</td>
</tr>
<tr>
<td>4.3.1 Genes overexpressed in the PrL</td>
<td>138</td>
</tr>
<tr>
<td>4.3.2 Genes overexpressed in the VO</td>
<td>148</td>
</tr>
<tr>
<td>4.3.3 Genes overexpressed in the Rt</td>
<td>152</td>
</tr>
<tr>
<td>4.4 Discussion</td>
<td>162</td>
</tr>
<tr>
<td>4.4.1 Regional expression analysis by LMD-microarray and ISH: technical considerations</td>
<td>162</td>
</tr>
<tr>
<td>4.4.2 Validation of genes overexpressed in the PrL</td>
<td>163</td>
</tr>
<tr>
<td>4.4.3 Validation of genes overexpressed in the VO</td>
<td>165</td>
</tr>
<tr>
<td>4.4.4 Validation of genes overexpressed in the Rt</td>
<td>166</td>
</tr>
</tbody>
</table>

### 5. Effect of Chronic PCP Treatment on Expression of Genes Enriched in the PrL, VO and Rt

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Introduction</td>
<td>169</td>
</tr>
<tr>
<td>5.1.1 Pharmacology of PCP</td>
<td>170</td>
</tr>
<tr>
<td>5.1.2 Psychotomimetic effects of PCP in humans</td>
<td>171</td>
</tr>
<tr>
<td>5.1.3 PCP models of schizophrenia</td>
<td>172</td>
</tr>
<tr>
<td>5.2 Aims</td>
<td>175</td>
</tr>
<tr>
<td>5.3 Results</td>
<td>176</td>
</tr>
<tr>
<td>5.3.1 Genes overexpressed in the Rt</td>
<td>176</td>
</tr>
<tr>
<td>5.3.2 Genes overexpressed in the VO</td>
<td>180</td>
</tr>
<tr>
<td>5.3.3 Genes overexpressed in the PrL</td>
<td>183</td>
</tr>
<tr>
<td>5.4 Discussion</td>
<td>190</td>
</tr>
<tr>
<td>5.4.1 GABA&lt;sub&gt;δ&lt;/sub&gt;</td>
<td>190</td>
</tr>
<tr>
<td>5.4.2 Wolfi'amin</td>
<td>191</td>
</tr>
</tbody>
</table>
6. Effect of Chronic PCP Treatment on Expression of Genes Enriched in the PR and RT That Have Chromosomal Location Linked to Schizophrenia

6.1 Introduction

6.1.1 Chromosomal loci linked to schizophrenia
6.1.2 Localised expression of genes with chromosomal location linked to schizophrenia
6.1.3 Localised expression of genes with chromosomal location 5q
6.1.4 Localised expression of genes with chromosomal location 22q
6.1.5 Localised expression of genes with chromosomal location 2p

6.2 Aims

6.3 Results

6.4 Discussion

7. General Discussion

7.1 Expression profiling in laser-microdissected brain regions
7.2 Confirmation of regional expression by in situ hybridisation
7.3 Expression of validated localised genes in the chronic PCP model of schizophrenia
7.4 Expression of genes with chromosomal location linked to schizophrenia in the chronic PCP model
7.5 Further work
7.6 Conclusions

References
1. Introduction
1.1 Schizophrenia

Schizophrenia is a psychiatric disorder that affects the most complex and developed functions of the human brain. Thought, language, communication, social interaction, motivation and cognitive functions are dramatically compromised in schizophrenic patients. This condition is differentiated from other diseases with a psychotic component, such as delusional disorder and schizoaffective disorder, on the basis of the combination of symptoms displayed by the patients and their duration. The American Psychiatric Association’s fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) defines schizophrenia as “a disturbance that lasts for at least 6 months and includes at least 1 month of active phase symptoms” including positive and negative symptoms (refer to sections 1.1.2 and 1.1.3). Also, subtypes of schizophrenia are classified according to the prevalence of some symptoms over others (refer to section 1.1.5).

The German physicist Emile Kraepelin first described the disorder currently known as schizophrenia according to modern scientific diagnostic criteria in 1887. Kraepelin named this condition “dementia praecox” (early dementia), to distinguish it from other forms of dementia that commonly occur in senescence, such as Alzheimer’s disease. The term “schizophrenia” was applied to the mental disorder described by Kraepelin by the Swiss psychiatrist Eugen Bleuler in 1911, who considered the definition “dementia praecox” misleading, as this condition is not always associated with mental deterioration. The word schizophrenia, which originates from the Greek words for “split” (σχισμός) and “mind” (νοῦς), was coined to describe the fragmented thinking of patients. This definition should not be referred to multiple or split personality, which is not a feature of schizophrenia.
Schizophrenia has a characteristic time course. The onset is typically in late adolescence-early adulthood. Clinical signs are not frequently detectable before puberty, though in some cases a prodromal phase of the illness might be observed. Prodromal features include cognitive and mood symptoms, social withdrawal and obsessive behaviours (McGlashan, 1996); however, they are not fully specific and lack diagnostic validity. Schizophrenia is a chronic disease, with untreated patients usually undergoing a gradual exacerbation of the illness, characterised by persistent and severe negative symptomatology and cognitive impairment (see sections 1.1.3 and 1.1.4) with typically frequent manifestation of positive symptoms (see section 1.1.2). Usually, the severity of the symptoms becomes stable in the senescence (Lewis et al., 2000).

1.1.1 Diagnostic criteria for schizophrenia

Schizophrenia is diagnosed according to the guidelines outlined in the DMS-IV and in the tenth revision of the International Classification of Diseases (ICD-10), which is the official diagnostic manual of the World Health Organisation (WHO). The DMS-IV is commonly used in the United States, whereas the ICD-10 is the prevalent manual employed by psychiatrists in Europe. Both publications describe the same characteristic symptoms outlining uniform diagnostic criteria; in fact scientific publications can refer to either manuals. Moreover, the diagnostic criteria outline exclusion cases to facilitate the differentiation between schizophrenia and other conditions with a psychotic component.

The core symptoms of schizophrenia can be divided into three main categories: positive symptoms, negative symptoms and cognitive impairment. The positive symptoms, which include delusions, hallucinations, thought disorder, disorganised behaviour and catatonic motor behaviour (see section 1.1.2 for detailed description) are an excess or distortion of a normal function, whereas the
negative symptoms, such as affective flattening and avolition (see section 1.1.3), consist of a diminution or loss of normal functions. When diagnosing schizophrenia it is essential that a number of core symptoms are shown for a significant portion of time during a 1-month period, and that signs of the disorder persist for at least 6 months (DMS-IV). The importance of this time scale is due to the presence of psychotic symptoms for a limited time in other disorders such as schizoplreniform disorder, schizoaffective disorder, delusional disorder, brief psychotic disorder and drug-induced psychosis. The diagnosis is complicated by the heterogeneity of schizophrenia; in fact different types of symptoms can be predominant in specific subsets of patients (see section 1.1.5). Also, although both the ICD-10 and the DMS-IV outline the diagnostic criteria in detail, the identification of the symptomatology relies on the patient’s verbal descriptions.

1.1.2 Positive symptomatology of schizophrenia

1.1.2.1 Delusions

Delusions are defined by the DMS-IV as “erroneous beliefs that usually involve a misinterpretation of perceptions or experiences”. Delusions may have many themes, sometimes depending on the cultural background of the patient. Persecutory delusions, including the belief of being tormented, followed, spied on or subjected to ridicule can be frequent, as well as referential delusions, such as the belief that gestures, passages from books or newspaper are specifically directed to oneself.

Interestingly, the presence of bizarre delusions is a relatively specific feature of schizophrenia, although it may be difficult to judge the bizarreness of false beliefs (DMS-IV). However, there are some examples of bizarre delusion that are common in the disease; these include the patient’s belief that his or her thoughts
have been taken away by an external force ("thought withdrawal"), that external thoughts are being inserted in his or her mind ("thought insertion") or that his or her actions are being directed by an outside force (delusions of control).

1.1.2.2 Hallucinations

Schizophrenic patients can experience hallucinations involving any sensory modality, although auditory hallucinations are by far the most common. Distorted perceptions are considered a symptom of schizophrenia only if they occur in a normal sensorial context, as opposed to hallucinations perceived while falling asleep (hypnagogic) or while waking up (hypnopompic). On the other hand, some specific types of auditory hallucination are considered specific to schizophrenia symptomatology. These include two or more external voices conversing with each other and an external voice keeping a running commentary on the patient’s actions (DMS-IV).

1.1.2.3 Thought disorder

Disorganised thinking is considered one of the core symptoms of schizophrenia. This characteristic is reflected by disorganised speech, and can be difficult to define, as a mild form of this symptom can be common and non-specific to schizophrenia. Thought disorder in schizophrenic patients can be very severe, impairing the patients’ communication skills and consequently their ability to interact in social environments. In some cases disorganised thinking can lead to incoherent and incomprehensible speech.
1.1.2.4 Disorganised behaviour

Disorganised behaviour usually manifests itself in schizophrenic patients as the inability to perform goal directed actions. Frequently patients are unable to organise activities that are necessary for everyday life, such as arranging meals or keeping personal hygiene; occasionally inappropriate sexual behaviour and unmotivated and unpredictable agitation can occur. It is worth noting that these symptoms are separate from behaviour emanating from delusional beliefs.

1.1.2.5 Catatonic motor behaviour

Catatonic motor behaviour is defined as a marked decrease in reactivity to the external environment (DMS-IV). In some cases this symptom can be very severe, resulting in a complete unawareness of the surrounding environment (catatonic stupor) that can be associated with rigid posture and resistance to attempts to be moved (catatonic rigidity). The assumption of unusual postures (catatonic posturing) and purposeless unstimulated excessive motor activity (catatonic excitement) can also be observed, especially in a subtype of schizophrenia (refer to section 1.1.5).

1.1.3 Negative symptomatology of schizophrenia

As mentioned previously, negative symptoms reflect a deficit in normal functions and are an important feature of schizophrenia that contribute substantially to the morbidity of this disease. The evaluation of negative symptoms can be difficult due to a number of confounding factors. For example
mood disorders and also side effect of antipsychotic medication can mimic affective flattening or avolition (see below).

1.1.3.1 Affective flattening

Affective flattening is a common symptom in schizophrenic patients. It is usually manifested as a distinctly reduced range of emotional expressiveness; for example the patients may show poor eye and body language when interacting with peers, coupled with inexpressive and unresponsive facial expression.

1.1.3.2 Alogia

Alogia is defined as a “decreased fluency and productivity of speech” (DMS-IV) that reflects impoverished thought. It is crucial to differentiate this symptom from the unwillingness to speak, which is not related to impaired thinking.

1.1.3.3 Avolition

Avolition is the “inability to initiate and persevere in goal-directed activities” (DMS-IV). The patients showing this symptom usually show very little interest in participating in social activities and work. It is important to differentiate this symptom from side effects of antipsychotic drugs or behaviour that is dependent on delusions.
1.1.4 Cognitive dysfunction in schizophrenia

Cognitive impairments are disturbances in basic cognitive function such as attention, executive functions and specific forms of memory, particularly working memory (Lewis et al., 2000). These impairments are considered a core feature of schizophrenia (Park et al., 1992) and play a pivotal role in a patient's behavioural disturbances and functional disability; for example, social interaction is compromised by the difficulty in correctly perceiving and processing the emotions displayed by others both in facial expression and vocal communication (Edwards et al., 2002).

Impairments in working memory and attentional processes, which are linked to disturbances of the prefrontal cortex (Park et al., 1992; Goldman-Rakic, 1994) are particularly evident. Schizophrenic patients perform poorly in tests specifically designed to assess executive function, such as the Wisconsin Card Sort Test (WCST) (Park, 1997) and spatial working memory, such as the oculomotor delayed response tasks (Park et al., 1992). Briefly, in the WCST the subject is required to flexibly decipher and apply rules concerning the arrangements of cards. The test involves sorting the cards according to a specific rule, such as shape or colour; once a pattern is established, the subject is asked to arrange the cards applying a different rule. The capability to maintain and shift attention and to formulate abstract concepts (behavioural flexibility) is crucial for performance in the WCST. Schizophrenics also perform poorly in the classic Tower of Hanoi test (Goldberg et al., 1990). This is another task used to assess executive function, and requires the subject to rearrange a tower of discs of decreasing sizes with the fewest moves.

It has been shown that cognitive impairments are present even in first-episode previously untreated patients, suggesting that these symptoms are an intrinsic characteristic of schizophrenia, rather than the consequence of prolonged neuroleptic treatment (Bilder et al., 2000). Moreover, an attenuated form of the cognitive impairments can be detected in the prodromal phase of the illness,
before the onset of other symptoms (Jones et al., 1994), as well as in non-schizophrenic relatives of patients (Cannon et al., 1994).

Cognitive impairments have great importance in the complex psychopathology of schizophrenia, and, similar to negative symptoms, are insufficiently ameliorated by drug therapy. The persistence of severe cognitive impairment is a determinant factor for progression of the disease and complicates the re-integration of patients in social activities and work environment.

1.1.5 Subtypes of schizophrenia

Subtypes of schizophrenia are differentiated according to the predominant symptomatology at the time of diagnosis. Importantly, the clinical characteristics of the disorder may change over time and in some cases patients may show symptoms that are representative of different subtypes of schizophrenia.

1.1.5.1 Paranoid schizophrenia

Paranoid schizophrenia is characterised by the prevalence of positive symptoms such as delusions and hallucinations. Delusions may be multiple and bizarre, but they are usually organised coherently. Hallucinations can also be related to the context of delusions. Frequently, patients suffering from paranoid schizophrenia can be anxious, aloof or irritable due to typical persecutory theme of their delusions. Disorganised speech, avolition and affective flattening are not predominant in this subtype of schizophrenia; moreover, cognitive impairments are not severe. Usually paranoid schizophrenia has a considerably better prognosis than other subtypes, possibly due to the relatively intact cognitive function of this
subclass of patients. Also, positive symptoms can be controlled by treatment with antipsychotics.

1.1.5.2 Disorganised/hebephrenic schizophrenia

Disorganised schizophrenia, historically known as hebephrenic, is characterised by the prevalence of thought disorder, disorganised behaviour and affective flattening. In hebephrenic patients, the capacity of performing goal-directed actions is severely impaired. This clinical profile frequently causes serious disruption of activities necessary for everyday and working life such as, organising meals, keeping personal hygiene, performing in a work environment. Cognitive impairment is also a core feature of this subtype of schizophrenia, which is usually associated with poor prognosis and early onset.

1.1.5.3 Catatonic schizophrenia

When catatonic motor behaviour is marked, schizophrenia is classified as catatonic even if other symptoms are present. The psychomotor disturbances (refer to section 1.1.2.5) can represent a risk factor for malnutrition, exhaustion and self-harming; therefore catatonic patients usually require adequate supervision and are usually hospitalised.

1.1.5.4 Undifferentiated schizophrenia

Schizophrenia is classified as undifferentiated when the symptoms shown by the patients correspond to the core features of the disease, but do not meet the criteria to be diagnosed as paranoid, disorganised or catatonic schizophrenia. This
situation may occur when there is no clear prevalence of one type of symptomatology.

1.1.5.5 Residual schizophrenia

Residual schizophrenia is usually a transition period between the acute phase of the disease and a complete remission, although it could be persistent for several years with or without relapses. It is characterised by negative symptoms such as affect flattening and avolition. Positive symptoms are absent or very attenuated.

1.1.6 Pharmacotherapy of schizophrenia

Early pharmacological treatments of schizophrenia were based on sedation of agitated patients and included administration of drugs such as morphine, potassium bromide, chloralum hydrate and hyoscine (Shorter, 1997). Sleep therapy induced by a combination of barbiturates and insulin coma were also common treatments until the mid-1950s (Ban, 2004). While these approaches were undoubtedly effective to control agitation and aggressive behaviour of some patients, they had very marginal, if any, benefit for the remission of the symptoms of schizophrenia.

Chlorpromazine was the first drug with relatively specific antipsychotic properties that allowed the treatment of schizophrenia whilst avoiding generalised non-specific sedation. This compound was synthesized and introduced for clinical use in the early 1950s (Charpentier et al., 1952; Delay et al., 1952). A few years later, haloperidol, which is still commonly used, was introduced (Divry et al., 1959). The introduction of these drugs in the therapy of psychosis not only
improved the conditions of patients, but also prompted the development of modern neuropsychopharmacology. Importantly, the finding that these antipsychotics are antagonists of the dopamine D2 receptor (Creese et al., 1975) led to the development of the dopamine hypothesis of schizophrenia (Snyder, 1976) (see section 1.3.1). Unfortunately, the blockade of dopamine receptors also induces severe extrapyramidal manifestations, such as bradykinesia and tardive dyskinesia, which are the most prominent side effects of this class of drugs (Farde et al., 1992). Other limitations of chlorpromazine, haloperidol and other drugs with similar pharmacological profile include limited beneficial effect for the negative symptoms and cognitive impairments of schizophrenia and the fact that a relatively high proportion of patients do not respond to the treatment (Kane, 1987).

The introduction of a second generation of antipsychotics including clozapine, olanzapine, quetiapine and risperidone provided the psychiatrists with a wider range of drugs to treat the symptoms of schizophrenia, and contributed to the revision of the postulate that the effectiveness of this class of drugs is dependent on their dopamine D2 receptor antagonism. The pharmacology of these compounds is complex, as they interact with several receptors in the central nervous system, including serotonin 5-HT2A; however they usually lack prominent anti-D2 activity, opposed to the first generation of antipsychotics. These drugs, also classified as atypical antipsychotics, do not induce prominent extrapyramidal side effects. Despite this advantage, these drugs represent only a limited improvement in the treatment of schizophrenia compared to the typical, first-generation antipsychotics, as many key symptoms such as the cognitive disturbances (see section 1.1.4) remain untreated (Davis et al., 1980). Moreover, refractory patients do not respond or respond only partially to pharmacological treatment regardless of which class of antipsychotics is used (Kane, 1987; 1996).

Although substantial advances since the early pharmacotherapeutic approaches have significantly improved the conditions of schizophrenic patients, the drugs currently available are certainly not sufficient to effectively resolve the symptomatology of this disease. While some subtypes of the disease, such as
paranoid schizophrenia, respond relatively well to antipsychotics, treatment of patients with prevalent cognitive impairments is less likely to be successful. Improvement of the understanding of the neurobiological basis of cognitive deficits could contribute to the development of novel pharmacological treatments to ameliorate this core symptom of schizophrenia.

The heterogeneity of schizophrenia is another important factor that needs to be considered for the development of innovative pharmacotherapeutical approaches. Subpopulations of patients are likely to respond differently to different combinations of drugs; therefore improved diagnosis coupled with specific therapeutic approach could be one of the goals for the future generation of treatments (Ban, 2004).
1.2 The neuropathology of schizophrenia

The attempt to identify schizophrenia-linked neuropathological alterations that can be reliably measured has been a major challenge for the neuroscience community since this disease was first described. In fact, Kraepelin was convinced that the syndrome he identified as dementia praecox one century ago was an organic brain disease, although the diagnostic criteria were, and still are based on the assessment of the psychopathological profile. For a long time the efforts to categorize measurable pathological changes in the schizophrenic brain have been frustrated by a number of factors, including inadequate techniques. The inconsistency of the data collected before the development of CT (computed tomography) and MRI (magnetic resonance imaging) are reflected by the infamous statement that schizophrenia is the “graveyard of neuropathologists” (Plum, 1972). The new imaging techniques, coupled with improved methodologies for post-mortem studies have allowed some of the difficulties that affected the earlier studies to be partially overcome. Recent consistent findings have prompted a new enthusiasm for the investigation of the neuropathological changes in the schizophrenic brain and appear to support that schizophrenia is an organic brain disease (Ron et al., 1990; Weinberger, 1995), as initially postulated by Kraepelin. Nevertheless, some data are still contradictory (see below) and further studies are needed to clarify whether some neuropathological alterations are shown in specific subsets of patients only. The clinical differences between various schizophrenia subtypes can be an obstacle to the identification of a general neuropathological profile; therefore the diagnostic criteria must still rely on the psychopathology, taking little advantage of the limited consistent evidence available about the organic alterations of the schizophrenic brain.
1.2.1 Macroscopic alterations in the schizophrenic brain

1.2.1.1 Whole brain volume

A number of studies report a statistically significant decrease in whole brain volume of schizophrenic patients compared to control subjects; however these data have been contradicted by several reports of negative findings (reviewed by Shenton et al., 2001). Despite the inconsistency of data regarding decreased whole brain volume in schizophrenia, it is possible that small changes are associated at least to some subsets of cases. Statistically significant reduced brain volume has been demonstrated in patients with childhood schizophrenia (Jacobsen et al., 1996), suggesting that this neuropathological abnormality could be specifically associated with early-onset forms of the disease, as concluded by Shenton et al., 2001.

It is also important to consider that the size of head and brain is very variable in the general population, and can be influenced by factors such as age, gender and nutritional deficits. This variability can be a confounding factor when studying small but important changes.

1.2.1.2 Size of ventricles

Enlargement of the lateral ventricles is one of the most consistent macroscopic alterations measured in the schizophrenic brain (Daniel et al., 1991; van Horn et al., 1992). The development of imaging techniques such as CT and MRI has been crucial to measure the ventricular size in a reliable and non-invasive way, leading to interesting findings. Yotsutsuji et al. (2003) showed that the ventricular enlargement is particularly evident in the temporal horn portion of the lateral ventricles using high-resolution three-dimensional MRI. Interestingly, these findings are concordant with data from post mortem analyses (Roy et al., 1998;
Nieman et al., 2000), confirming the consistency of this neuropathological change.

The ventricular enlargement is associated with a loss of brain tissue, suggesting that the reduction of grey matter volume in the region surrounding the temporal horn of the lateral ventricles is a feature of schizophrenia (Lawrie et al., 1996).

Unfortunately the enlargement of the lateral ventricles cannot be considered as a specific feature of schizophrenia, as this abnormality can also be observed in other conditions, such as Alzheimer's disease and Huntington's chorea (Brown et al., 1986; Wang et al., 2002).

1.2.1.2 Temporal lobe

The temporal lobe is another brain region that shows robust changes in the schizophrenic brain. Interestingly, Kraepelin (1919) believed that some symptoms such as delusions and hallucinations could be related to neuropathological alterations of the temporal lobe. Almost a century later, technological advances in neuroimaging allowed demonstration of this intuition. Barta et al., (1990) and Shenton et al. (1992) reported that the severity of auditory hallucinations and thought disorder could be correlated with a decrease in size of the superior temporal gyrus. Other studies, however, show no significant ventricular or cortical size change (Dwork, 1997; Heckers et al., 1990). It is worth considering that in many cases contradictory results can be due to methodological differences between studies. Crucial factors that can influence measurements are the thickness of slices, the landmarks used to define boundaries between lobes, and the choice of measuring grey matter or grey matter and white matter combined (reviewed by Shenton et al., 2001).
1.2.1.3 Frontal lobe and limbic system structures

The frontal lobe includes structures that are crucial for the neuropathology of schizophrenia, such as the prefrontal cortex (PFC). Neuroimaging studies in human brain and lesion studies in non-human primates have demonstrated the correlation between PFC activation and working memory, (reviewed by Honey et al., 2006), which enables the short-term retrieval of information for goal-directed action. Working memory is central to everyday functioning and planning, which are severely disrupted in schizophrenia, and also contributes to other cognitive processes. Consistent data show that many aspects of the cognitive deficits observed in schizophrenia (see section 1.1.4) are due to working memory impairment and are associated with dysfunctional PFC (Goldman-Rakic, 1994; Honey et al., 2006).

Despite the well-documented functional abnormalities in the schizophrenic prefrontal cortex, the available data about structural changes in the frontal lobe are contradictory (DeLisi et al., 1991; Lawrie et al., 1999; Sanfilipo et al., 2000). Interestingly, studies that did not find significant frontal lobe volume changes in the schizophrenic brain, reported a correlation between the volume of this area and structures of the limbic system in the schizophrenic brains only (Wible et al., 1995). The same study also reported a correlation between the severity of negative symptoms and frontal lobe volume reduction. Breier et al. (1992) also found a correlation between frontal lobe and temporal lobe volume in schizophrenia. Although these findings do not prove that the prefrontal dysfunction in schizophrenia is correlated to structural changes, they suggest that some macroscopical neuropathological alterations may exist but are difficult to detect. It is worth considering that the majority of studies on frontal lobe neuropathology have been conducted measuring the volume of the whole lobe (Shenton et al., 2001), although this brain area is not functionally and anatomically uniform. Perhaps alternative approaches coupling high anatomical specificity with improved technology could reveal evidence of prefrontal neuropathological alterations in the near future.
1.2.1.4 Other brain regions

MRI and CT studies have been performed in a number of non-cortical brain structures. The thalamus has been the focus of many investigations for its importance in the modulation of cortical activity. In fact, the ventral anterior and dorsomedial thalamic nuclei are crucial relay stations that establish reciprocal connections with the prefrontal cortex, suggesting that these brain subregions may play a role in schizophrenia (Andreasen et al., 1994). In fact, it is known that thalamic activity is important for filtering the sensorial input and controlling attentional processes (Jones, 1985; Fuster, 1989). Interestingly, there is evidence of neuropathological alteration in the thalamus from post mortem studies (Pakkenberg, 1987; Pakkenberg, 1990). Reports from imaging studies are more contradictory, partially due to the technical difficulties encountered when applying this technique to measure thalamic nuclei (Andreasen et al., 1990). Nevertheless, significant reduction of signal intensity in the thalamus of schizophrenic brains was reported by Andreasen et al. (1994). Moreover, a study reporting no significant difference between schizophrenic thalamus and control, noted a correlation between early onset of the disease and decreased thalamic volume (Corey-Bloom et al., 1995).

Neuropathological abnormalities were also reported in other brain structures such as the basal ganglia, the corpus callosum, the olfactory bulbs and the cerebellum (Turetski et al., 2003; Mamah et al., 2006; Brambilla et al., 2005). Interestingly, the cerebellum establishes connections with the cortical association areas and limbic structures such as hippocampus and amygdala, suggesting that this area contributes to cognitive processing (Schmahmann, 1996); hence the cerebellum may be an interesting candidate for investigating schizophrenia-related neuropathological abnormalities. Significant increase in white matter volume of the cerebellar vermis was reported by Levitt et al. (1999); the same study also noted a correlation between this change and the severity of positive symptoms, thought disorder and impaired verbal memory. Increased white matter volume in
the cerebellum can be interpreted as a sign of abnormal neurodevelopment (Levitt et al., 1999).

Evidence of macroscopic abnormalities in the schizophrenic brain is progressively more convincing thanks to the development of non-invasive imaging techniques such as MRI. The most substantial data show significant neuropathological changes in the lateral ventricles, temporal lobe, frontal lobe and subcortical regions such as thalamus, cerebellum, basal ganglia and corpus callosum (see above). The large number of regions involved in the neuropathological changes associated with schizophrenia suggests that this disease affects the connectivity of multiple areas which are likely to be functionally related. This scenario is compatible with the hypothesis that the abnormalities observed in the schizophrenic brain have a neurodevelopmental origin (for review see Weinberger, 1995). However, it is worth mentioning that the macroscopic alterations alone are not likely to explain the complex dysfunctions shown by schizophrenic patients; thus this type of information needs to be supported by data showing cellular and molecular alterations (for review see Harrison, 1999).

1.2.2. Cytoarchitectural abnormalities in schizophrenia.

Many studies are aimed at investigating the microscopic neuropathology of schizophrenia. Understanding the possible pathological changes at the neuronal level may be very relevant to the aetiology of this disease, as neurodegeneration is not commonly associated with schizophrenia (Harrison et al., 1999). The cytoarchitecture studies usually analyse the brain at the cellular and subcellular level, taking into account a number of neuronal characteristics that could change
in pathological conditions, such as number, density, size and morphology of neurons.

The focus has been mainly on the extended limbic system, whose activity is essential for higher cognitive and affective functions. Abnormalities in the cytoarchitecture and lamination of the entorhinal cortex (anterior parahippocampal gyrus) (Jakob et al., 1986), disarray of hippocampal pyramidal neurons (Kovelman et al., 1984) and abnormal location of cortical subplate neurons (Akbarian et al., 1993) have been reported; however other groups failed to replicate these findings (Akil et al., 1997; Altshuler et al., 1987; Anderson et al., 1996).

Decreased cellular size affecting neurons in the hippocampus and the dorsolateral prefrontal cortex (DLPFC) has been shown fairly convincingly (Harrison 1999). Image analysis techniques were employed to measure the size of the cell bodies, demonstrating that hippocampal pyramidal neurons have a smaller mean size in schizophrenia (Zaidel et al., 1997), as well as lamina IIIc neurons in DLPFC (Rajkowska et al., 1998). Some studies suggest that size reduction is accompanied by increased neuron density (Harrison 1999).

The other relatively robust cytoarchitectural abnormality found in schizophrenia is in the dorsal thalamus, which is smaller and contains fewer neurons (Danos et al., 1998; Pakkenberg, 1990; Pakkenberg, 1992). Interestingly, the neurons in mediodorsal nucleus of thalamus are reciprocally connected with prefrontal cortex neurons, consistent with the hypothesis that schizophrenia is caused by pathological changes of neuronal circuitry.

It has also been demonstrated that pathological abnormalities affect specific sub-cellular structures such as axons, dendrites and synaptic terminals. Synaptic protein levels are lower in the hippocampus and parahippocampal gyrus (Eastwood et al., 1995); these changes particularly affect the excitatory pathways of this region, supporting the involvement of the glutamatergic system in the neurochemistry of schizophrenia (see below). Studies performed on the DLPFC have found lower density of dendritic spines on layer III pyramidal neurons (Garey et al., 1998) as well as lower expression of synaptophysin (Glantz et al.,
1997), a marker of synaptic density. Evidences of synaptic abnormalities have also been shown in the thalamus, where the synaptic protein rab3a is significantly reduced (Blennow et al., 1996), and in the striatum, where altered sizes of caudate nucleus synapses were observed with electron microscopy (Harrison, 1999).

Synaptic studies highlight the impaired connectivity affecting the schizophrenic brain. The regions involved are pivotal to most of the complex functions disrupted in schizophrenia; the changes found in the hippocampal formation confirm the involvement of the limbic system together with other regions anatomically and functionally interconnected such as the thalamus and the neocortex. The pathological changes primarily affect glutamatergic excitatory pathways in the hippocampus and the DLPFC, although the GABAergic system is also clearly involved and its role is important for the modulation of synaptic connectivity between the affected regions (see below).
1.3 Neurochemical abnormalities in schizophrenia

Schizophrenia is also characterized by specific changes in neurotransmission systems. Most of the research about schizophrenia neurochemistry has been inspired by pharmacology: the mechanisms of action of both antipsychotic and psychotomimetic compounds form the basis of the neurotransmitter hypothesis of schizophrenia.

1.3.1. Dopamine hypothesis.

Many studies focus on a possible alteration of dopaminergic transmission in schizophrenia. The dopamine hypothesis is supported by the fact that antagonists of dopamine receptors such as haloperidol and chlorpromazine are effective for the treatment of the positive symptoms of schizophrenia. Also, it is well known that amphetamine, which stimulates the release of catecholamines from synaptic terminals, can mimic the positive symptoms of schizophrenia in healthy individuals and exacerbates pre-existing psychosis in schizophrenic patients (Connell, 1958; Angrist et al., 1974). The psychotomimetic effect of amphetamine is consistent with the hypothesis that schizophrenia could be associated with hyperactivity of the dopaminergic system.

Analysis of dopamine receptor distribution and expression within the affected regions of the brain attempts to confirm the dopamine hypothesis. Dopamine receptors are classified into two classes: D1-like receptors, namely D1 and D5, which activate adenylyl cyclase via Gs coupling; and D2-like receptors, including D2, D3 and D4, which couple via Gi/o to inhibit adenylyl cyclase. The latter are particularly important in schizophrenia, since typical antipsychotics are antagonists of the D2 receptor. It has been shown that D2 receptor subset densities
are increased in the schizophrenic brain, but it is not clear whether this change is
due to pharmacological treatment or to the illness itself (Nordstrom et al., 1995;
Zakzanis et al., 1998). Data on D₁ and D₃ receptors alterations are controversial
(reviewed by Harrison 1999), as are those about the D₄ receptor, which is
particularly interesting being one of the sites of action of clozapine, an effective
atypical antipsychotic. Abnormal dopamine metabolism has also been
demonstrated (Elkashef et al., 2000), but pharmacological treatment cannot be
excluded as a cause of this change (reviewed by Wong et al., 2003). Interestingly
positron emission tomography (PET) and single photon emission tomography
studies show an increased release of dopamine from synaptic terminals in
response to amphetamine in schizophrenia, suggesting a hyperresponsiveness of
dopaminergic neurons (reviewed by Harrison 1999).

Dopaminergic hyperactivity has been considered for a long time as the main
cause of schizophrenia; indeed there is a correlation between dopamine D₂
receptor binding and clinical efficacy of typical antipsychotic drugs (Creese et al.,
1976). However, the attempts to demonstrate a specific molecular alteration in the
dopaminergic system have not yielded definitive results as yet. Furthermore the
dopamine hypothesis is not sufficient to explain all the complex features of
schizophrenia: amphetamine does not mimic the negative symptoms and cognitive
impairment, and effective atypical antipsychotic such as clozapine and risperidone
are not solely antagonists of the D₂ receptor. Nevertheless, dopamine is likely to
play an important role in schizophrenia pathology and should be considered as
one of the components of a complex scenario involving other neurotransmission
systems and neuropathological alterations.
1.3.2. Serotonergic system involvement.

Lysergic acid diethylamide (LSD)-induced hallucinations and behaviour resemble some symptoms of schizophrenia (Breier, 1995). As this drug is a 5-HT agonist, it has been hypothesised that the serotonergic system may play a role in the pathology of schizophrenia. Overexpression of the 5-HT$_{1A}$ receptor has been demonstrated in the frontal cortex of schizophrenic patients, while the expression of 5-HT$_{2A}$ receptors in the same region of the affected brain is decreased (Burnet et al., 1996). Interest centres on a possible role of 5-HT$_{2A}$ receptors because it is one of the sites of action of several atypical antipsychotics, such as clozapine, risperidone, olanzapine and quetiapine. These drugs have a complex pharmacological profile and interact with many different classes of receptor, including serotonergic, dopaminergic, histaminergic, adrenergic and muscarinic receptors. Their affinity for the D$_2$ receptor is usually lower than typical antipsychotics, so they cause less side effects connected with dopaminergic system inhibition, such as extrapyramidal effects (Seeman et al., 1997). At the same time, they are slightly more effective in ameliorating negative symptoms and cognitive impairment (Moller, 2003). For these reasons, a clarification of the role of the serotonergic system in schizophrenia and its interaction with dopaminergic and glutamatergic pathways would be an important achievement.

1.3.3. Glutamate hypothesis.

Glutamate is the main excitatory neurotransmitter in the brain, and it binds both to metabotropic and ionotropic receptors. The excitatory signalling is principally mediated by ionotropic receptors, which are classified into three classes named after their specific ligands: AMPA (amino-hydroxy-5-methyl-4-isoxazole propionic acid), kainate (kainic acid) and NMDA (N-methyl-D-aspartic acid). Phencyclidine (PCP), mainly a NMDA non-competitive antagonist, induces
a psychotic response that closely resembles the symptoms of schizophrenia (see chapter 5) (Luby et al., 1959). In contrast to amphetamine and LSD, PCP can mimic not only positive symptoms, but it can also cause negative symptoms and cognitive impairment, producing a psychosis so close to schizophrenia that is diagnostically difficult to differentiate (Olney et al., 1999). Ketamine, another NMDA antagonist, induces an array of symptoms resembling schizophrenia more frequently in adults than in children; this compound interestingly mimics the age-dependent vulnerability to the illness (Farber et al., 1995). Consequently, a hypofunction of NMDA receptors has been proposed as an interesting model of the disease.

Some changes of the glutamatergic system have been reported in schizophrenia: a decreased concentration of glutamate in the cerebro-spinal fluid (Kim et al., 1980) and altered expression of AMPA and KA receptors in the prefrontal cortex and hippocampus (Deakin et al., 1989; Kerwin et al., 1990). No consistent abnormalities in NMDA receptor density have been shown, but the subunit composition has been shown to be altered in the hippocampus, where a relative decrease of the NR1 subunit has been reported (Gao et al., 2000). NMDA receptors lacking the NR1 subunit are non-functional, so this finding suggests a hypofunction of glutamatergic system in the hippocampus (Goff et al., 2001).

Although NMDA receptors are not the main site of action for antipsychotics, recent research reports that some drugs affect glutamatergic neurotransmission. Clozapine, but not haloperidol, indirectly enhances NMDA-receptor-mediated neurotransmission in rat prefrontal cortical neurons in vitro (Arvanov et al., 1997). Indeed both clozapine and the selective 5-HT2A antagonist M100907 interfere with the PCP-induced blockade of NMDA receptors in rat prefrontal cortex (Wang et al., 1998), suggesting a role for serotonergic system in the NMDA hypofunction model. Furthermore, it has been shown that clozapine may enhance the glycine modulatory site occupancy on NMDA receptors; the occupation of this site is necessary for the glutamate-mediated activation of the ionotropic receptor (Coyle et al., 2002). These findings suggest that the ability of atypical antipsychotics to ameliorate negative symptoms and cognitive behaviour...
could be related to their interaction with the glutamatergic system (Goff et al., 2001). Dopaminergic neurotransmission also seems to be involved in the modulation of NMDA receptors: the D₂ receptor blockade by antipsychotic drugs promotes the expression of the NR₁ subunit of NMDA receptor in rats (Leveque et al., 2000).

For many years the focus of schizophrenia research has been on dopaminergic hyperfunction and anti-D₂ activity of antipsychotics. However the dopamine hypothesis does not explain important features of the disease such as the negative symptoms and cognitive impairments; in fact amphetamine only induces positive psychotic symptoms, and typical D₂ antagonist are not effective in ameliorating negative symptoms or cognitive deficits. The glutamate hypothesis is considered more useful than the dopamine hypothesis for several reasons: the PCP-induced psychosis closely resembles schizophrenia; and the administration of this drug to animals produces a robust model of the disease (see section 5.2). In particular the glutamate theory and PCP models are very useful to study negative symptoms and cognitive impairment, and they might help to identify new pharmacological targets for improved therapy. Nevertheless, it is unlikely that changes in glutamatergic neurotransmission alone are the cause of such complex symptoms and macroscopic alterations: D₂ antagonism is still important for the effectiveness against positive symptoms, and atypical drugs such as clozapine have a multi-receptor pharmacological profile. Many experimental evidences support the hypothesis of a complex neuropathological scenario, where the glutamatergic system is central, but structural changes and interactions with other neurotransmission systems must also be considered.
1.3.4. GABAergic neurons: their interaction with dopaminergic and glutamatergic systems and role in schizophrenia.

The excitatory function of glutamatergic neurons can regulate inhibitory pathways by synapsing on serotonergic, noradrenergic and GABAergic neurons, which express NMDA receptors. Furthermore, some GABAergic neurons have an inhibitory feedback function, so the glutamatergic primary neurons can regulate their own firing through the activation of NMDA receptors on the GABAergic interneurons (Olney et al., 1999). In such a model, an impairment of NMDA-receptor-mediated neurotransmission simultaneously affects inhibitory control over multiple excitatory pathways in relevant regions of brain (Olney et al., 1999).

GABAergic neurons also express dopamine receptors and could be indirectly involved in the action of antipsychotics. Specifically D4 receptors, one of the pharmacological targets of clozapine, are principally located on GABAergic neurons including the chandelier cell subset (see below) (Mrzljak et al., 1996).

Reduced activity and expression of glutamic acid decarboxylase (GAD67), the synthetic enzyme for GABA, have been demonstrated in the PFC of schizophrenic brains (Akbarian et al., 1995). GAD65, another isoform of the glutamic acid decarboxylase, also shows reduced expression in the cerebellum of schizophrenic patients; however this alteration is also present in bipolar disorder and depression (Fatemi et al., 2005). Impaired re-uptake and release of GABA in schizophrenia (Reynolds et al., 1990) confirm the importance of the GABAergic system. Interesting changes have been found in a particular subset of GABAergic neurons named chandelier cells. These neurons regulate the excitatory output of pyramidal glutamatergic neurons in schizophrenia-related regions of the brain such as the PFC. Their modulatory action is particularly effective as they synapse only on the axon initial segment of pyramidal neurons, close to the generation site of the action potential (DeFelipe et al., 1985). A decrease in the density of chandelier cell axons in the PFC has been shown in schizophrenia (Lewis, 2000).
suggesting that impaired inhibitory control by specific GABAergic neurons could be pivotal in schizophrenia neurochemistry. Moreover it has been shown that expression of parvalbumin, a calcium-binding protein localized in the chandelier cells, is lower in the PFC of schizophrenics (Beasley et al., 2002; Hashimoto et al., 2003).

Recent findings on GABAergic system involvement in schizophrenia enlarge the outlook on the complex neurochemistry of this disease. It is becoming more and more clear that the glutamatergic hypothesis has to be integrated with information about other neurotransmission pathways that could influence the neural circuitry affected by schizophrenia. In this scenario GABAergic neurons, particularly the chandelier subset, play a key role in the modulation of excitatory transmission both within and between brain regions affected by neuropathological abnormalities, such as prefrontal cortex and thalamic nuclei (Lewis, 2000). The studies performed by Lewis and co-workers suggest that schizophrenia is associated with a specific dysfunction of the connectivity between inhibitory chandelier cells and excitatory pyramidal neurons in the PFC (reviewed by Lewis et al., 2005). The evidence supporting this hypothesis include reduced expression of GAD67, GABA transporter 1 (GAT1) and parvalbumin in the chandelier cells of schizophrenic PFC, while the expression of these genes remain unchanged in other subtypes of inhibitory interneurons (Hashimoto et al., 2003). Moreover, Pierri et al. (2003) showed that the expression of the α2 subunit of the GABAA receptor is increased in the axon initial segment of pyramidal neurons in schizophrenic PFC, where chandelier cells establish synaptic connections. The upregulation of GABAAα2 could be an adaptative response to compensate the deficient release of GABA from the chandelier cells synaptic terminals (Lewis et al., 2005).
1.4 Animal models of schizophrenia

1.4.1 Modelling prefrontal cortex dysfunction in rats: limitations and advantages

One of the difficulties that schizophrenia research has to face is the limited availability of post mortem human tissue. Moreover even when the samples size is large enough, questions usually arise about the possibility that the results are influenced by the long pharmacological treatment that patients usually undergo. Indeed long years of therapy and superimposed pathologies or ageing can make the interpretation of data very complex. For these reasons, animal models of schizophrenia are very useful to provide the right amount of tissue for the studies under rigorously controlled conditions.

On the other hand, animal models of schizophrenia, and, more generally, of psychiatric diseases, are very difficult to assess. The first obvious reason is that schizophrenia pathology involves the most developed functions of human brain, such as complex social interactions, perceiving and expressing emotion, thought and language. These functions are less developed or completely absent in other mammals, included the commonly used experimental animals, such as rodents. Another reason is the lack of knowledge about the aetiology of schizophrenia. In fact it is very difficult to provoke in healthy animals a syndrome of which we do not know the pathological basis.
1.4.1.1 The prefrontal cortex in humans and rodents: anatomical heterogeneity and functional homologies

The complexity of schizophrenia is reflected by the number of brain regions that have been associated with this disease in studies investigating neuropathological, neurochemical and functional alterations (see sections 1.3 and 1.4). These findings suggest that the symptomatology of schizophrenia is dependent on multiple alterations in different brain regions. Nevertheless, it is clear that the dorsolateral prefrontal cortex play a key role in this disease (see sections 1.3 and 1.4), therefore it is necessary to consider whether it is realistic to model human prefrontal cortical function in animals.

The anatomical and functional correspondence between human and rodent brain structures has often generated controversies in the scientific community, especially when concerning regions responsible for the higher primate-specific functions (Brown et al., 2002). In fact, the prefrontal cortex is arguably the most unique region to the human brain, as it underlies the most evolved functions such as attentional control, decision-making, and temporal organisation of behaviour (Shallice et al., 1998; Damasio, 1998; Fuster, 2000).

Anatomically, it is evident that there are important differences between the primate brain and the rodent brain at the level of the dorsolateral prefrontal cortex. In primates, this region is defined by the incoming projections from the mediodorsal thalamic nucleus (Rose et al., 1948). In rats, the mediodorsal nucleus projects to the medial and orbital prefrontal cortex, but not to the dorsolateral regions; therefore rats do not have a brain region that matches the human dorsolateral prefrontal cortex by neuroanatomical criteria only (Brown et al., 2002). On the basis of this observation, one could disregard any research attempting to use rodents as a model of higher cognitive functions or psychiatric conditions that involve the dorsolateral prefrontal cortex, such as schizophrenia. Nevertheless, comparative neuroscience between primates and rodents cannot be limited to the mere neuroanatomy, but must also take into account functional
analogy and homology. In this specific case, it is necessary to question whether the rat medial and orbital prefrontal cortex may underlie functions that are equivalent to those localised in the human dorsolateral prefrontal cortex. If the rat shows complex behaviour that would require the activation of the dorsolateral prefrontal cortex in primates, then such a functionally homologous region should exist in the rodent brain (Brown et al., 2002).

Modelling prefrontal cortical functions in rat is very difficult for a number of reasons, including obvious species-specific features such as language and verbal reasoning. Also, it is important to specifically adapt the tests to the species in order to analyse the cognitive substrate of behaviour. Many studies in the literature are designed to analyse how the prefrontal cortex affects processes dependent on working memory. Interestingly, it has been demonstrated that specific damage to the rat medial prefrontal cortex causes behavioural deficits in working memory-dependent tasks that are similar to those observed in primates with lesioned dorsolateral prefrontal cortex (de Bruin et al., 1994; Joel et al., 1997; Kesner, 2000). These findings suggest that, similarly to primates, behavioural flexibility is severely affected in rats with lesions at the level of the prefrontal cortex.

Impaired behavioural flexibility is also thought to have a role in the attentional deficits shown by prefrontal cortex lesioned rats (Brown et al., 2002). The five-choice serial reaction time task is a popular test to measure selective attention in which the rat is presented a visual cue in one of five possible locations; a number of parameters such as the duration of the stimulus can be varied to modulate the attention load required for the task (Carli et al., 1983). This important experimental approach has been used to show the attentional deficits of rats with lesioned prefrontal cortex (Muir et al., 1996; Granon et al., 1998). Importantly, the impairments observed were not compatible with perceptual attention difficulties, but they suggested a deficit in behavioural flexibility (Miner et al., 1997).

Behavioural flexibility is a key function of the human dorsolateral prefrontal cortex that is relevant to the symptomatology of schizophrenia (refer to section
1.1.4). Deficits of this important function have been shown to underlie the impairments in reversal learning observed in rats with lesioned prefrontal cortex performing visual discrimination tasks (Bussey et al., 1997). In particular, these deficits seem to affect the behaviour based on “higher order rules”, which involves the abstraction of associations to formulate rules necessary for strategic goal-directed behaviour (Kesner, 2000). The Wisconsin Card Sort Test is one of the most important tasks employed to study the abstraction of higher order rules in humans; interestingly, schizophrenic patients show performance impairments in this test (Park, 1997). It is possible to use a formally equivalent attentional set-shifting task to analyse this type of behaviour in rats (Birrell et al., 2000). In this test rats are trained to search for a food reward that is hidden in a bowl under a digging medium. The animals are presented with two bowls, only one of which contains the reward. The animals are faced with a choice that has to be made on the basis of multiple parameters, such as odour, type of digging medium and texture. The test involves a series of sessions that allow analysis of reversal learning, intra-dimensional and extra-dimensional shift. This experimental approach led to the demonstration that rats, in a similar manner to monkey and humans, are able to process the stimuli in order to form higher order rules necessary for complex strategic behaviour (Birrell et al., 2000). It has been shown that specific lesions of the medial and orbital prefrontal cortex in the rat brain cause impairments of the attentional set shifting; in particular, the medial prefrontal cortex is directly linked to the extradimensional shift of the attentional set (attentional flexibility), whereas the orbital part is associated with reversal learning (Birrell et al., 2000) (see also section 3.1.1).

The observations discussed above suggest that although the rat brain does not have a region that is anatomically analogous to the human dorsolateral prefrontal cortex (Preuss, 1995), these animals show some aspects of the complex behaviour that requires the activation of this region in primates. Lesion studies clearly show that the medial and orbital subregions of the rat prefrontal cortex are necessary for these functions; therefore they may be considered at least partially equivalent to the primate dorsolateral prefrontal cortex from a functional standpoint (Brown et
This information supports the study of rat prefrontal cortex as useful model of cognition, especially to complement research on schizophrenia, as the studies performed using human tissue can be affected by limited availability of material and several confounding factors (see above). Still, it is important to acknowledge that studies on the rat prefrontal cortex cannot aim to model the functions of the primate dorsolateral prefrontal cortex in a comprehensive way, but they rather constitute a simplified model of cognition with common key features that allow the extrapolation of useful information about human behaviour, cognition and pathological alterations (Brown et al., 2002).

Taking into consideration the issues discussed above, a number of different animal models of schizophrenia have been developed. None of them is able to mimic comprehensively the complex array of schizophrenia features, but animal models are undoubtedly successful in reproducing some important biological and behavioural characteristics of this illness. The schizophrenic-like alterations in animals are usually ameliorated by antipsychotic treatment; therefore animal models are particularly useful to study drug action and to predict new possible pharmacological targets.

Three criteria are usually considered to evaluate the suitability of an animal model: construct validity, face validity and predictive validity. Construct validity is the ability to emulate the aetiological basis of the disease; face validity criterion is defined as the capability to mimic the symptoms of human disease; predictive validity criterion is met when drugs that have established effectiveness against the human disease, are able to restore the physiological parameters in the animal model, whereas other classes of drugs should be inactive.
1.4.2. Environmental animal models of schizophrenia.

Some features of schizophrenia are reproduced in animals by specific environmental manipulation. The types of environmental stress used to model schizophrenia are usually designed to mimic some proposed aetiological factors for the disease; examples include isolation-rearing (Valzelli et al., 1977), pre-weaning non-handling (Shalev et al., 1998), hypoxia (Schwarzkopf et al., 1992) and prenatal maternal malnutrition (Morgane et al., 1993). Isolation-rearing produces a number of relevant changes such as enhanced DA-agonist induced stereotypic movement and spontaneous hyperactivity (Lillrank et al., 1995). Moreover, similarly to schizophrenic patients, isolated-reared animals show impaired pre-pulse inhibition (PPI) (Cilia et al., 2001). This test analyses deficits in sensorimotor gating, measuring the ability to modify a startle response to a loud tone. The startle response is normally attenuated by a low volume “warning sound”, the pre-pulse; this inhibition is clearly impaired in schizophrenic patients. Interestingly, the abnormalities caused by isolation-rearing are reversible by atypical antipsychotics (Bakshi et al., 1998).

Hypoxia and prenatal maternal malnutrition are also used to provoke schizophrenic-like behaviour. Consistent with the dopamine hypothesis these environmental models are associated with neurochemical changes in the dopaminergic system (Lillrank et al., 1995).

Environmental models are useful to investigate the neurobiological and behavioural changes caused by environmental stressors that could be involved in the aetiology of schizophrenia. Also, these animal models might be useful to analyse the gene-environment interactions and how stressors influence gene expression. Nevertheless, the construct validity of these models remains limited, since no environmental stressor alone has been proved to cause schizophrenia.
1.4.3 Lesion models of schizophrenia.

Lesion models are based on specific damage to brain regions that are crucial to the functions impaired in schizophrenia. Although this approach cannot reproduce the aetiology of the disease, lesion models can be useful to understand the importance of specific brain regions to cognition and behaviour. Also, models based on specific neuronal damage in the foetus and newborn animals are relevant to the neurodevelopmental aspect of schizophrenia.

An example of neuroselective induced toxicity relevant to schizophrenia is the foetal administration of methyl-azoxymethanol acetate (MAM) (Johnston et al., 1988). This compound selectively destroys rapidly dividing neurons, causing a disruption of the cytoarchitecture of the prefrontal cortex and the hippocampus. The behavioural abnormalities observed in this model include hyperactivity and cognitive impairment (Johnston et al., 1988). Interestingly, a recent study reported that the hippocampal parvalbumin-containing interneurons are decreased in the MAM model (Penschuck et al., 2006).

Another well-known lesion model with relevance to neurodevelopment is based on neonatal ventral hippocampal insult. This model mirrors some neurochemical abnormalities of schizophrenia. In fact, neonatal hippocampal lesions modify dopamine levels in key areas such as the prefrontal cortex and enhance dopamine-mediated behaviour (Lipska et al., 1992). This model is mainly relevant to the dopamine hypothesis of schizophrenia, and it can be used in combination with amphetamine exposure. The main behavioural alterations include hyperactivity, impaired pre-pulse inhibition and deficits in spatial learning and working memory (Lipska et al., 1995; Chambers et al. 1996). It is interesting that the behavioural abnormalities observed in the hippocampal lesion model are strain dependent, suggesting a relevance to the contribution of genetic factors to the neuropathology of schizophrenia (reviewed by Wong et al., 2003).

The medial prefrontal cortex is another target area for lesion model of schizophrenia. Specific excitotoxic lesions are induced by intracranial injection of
ibotenic acid in neonatal rats. Similarly to the ventral hippocampal lesion approach, this model reproduces alterations of the dopaminergic system (Flores et al., 1996). The medial prefrontal cortex and ventral hippocampal lesion models target two of the areas that are most affected in the schizophrenic brain (see section 1.3). It is important to note that these regions are reciprocally interconnected, therefore lesions in the hippocampus will influence prefrontal cortical function and vice versa (Carr et al., 1996).

1.4.4. Pharmacological models of schizophrenia.

The administration of psychotomimetics to animals induces biochemical and behavioural changes that can mimic those observed in the human psychiatric disease.

Pharmacological models of schizophrenia have extremely limited construct validity. Face validity is also limited, but a number of tests confirm schizophrenic-like behaviour and biological changes in these models. The third criterion, predictive validity, is often met, therefore pharmacological studies largely benefit from the analysis of these models.

1.4.4.1 Dopaminergic agents

Consistent with the dopamine hypothesis (see section 1.4.1), animal models based on the administration of direct or indirect dopamine agonists such as apomorphine and amphetamine are useful for predicting the activity of compounds against positive symptoms of schizophrenia. The face validity of dopaminergic models is supported by a number of behavioural tests. Dopamine agonists cause hyperlocomotion and stereotypy (Kokkinidis et al., 1980).
Although it could be argued that schizophrenic patients do not always show these
behavioural disturbances, antipsychotics attenuate amphetamine-induced
stereotypic behaviour. PPI is also disrupted in this model, as well as in
schizophrenics (Swerdlow et al., 1998). The PPI deficit parameter is corrected by
antipsychotics; furthermore this response is correlated both to antipsychotic
potency and D₂ receptor affinity (Marcotte et al., 2001).

Behavioural tests and antipsychotics action support the face validity and the
predictive validity of this dopamine model. Nevertheless, the hyperdopaminergic
state is not associated with negative symptoms in humans, therefore limiting the
usefulness of this model to the study of positive symptoms.

1.4.4.2 Serotoninergic agents

The effects of the two major classes of hallucinogenic drugs, indoleamines
(LSD) and phenethylamines (mescaline) are mainly due to their interaction with
5-HT₂A receptors. The psychotomimetic properties of these drugs in humans are
well known; moreover, the affinity of atypical antipsychotics such as clozapine
for the 5-HT₂A receptors supports the role of the serotoninergic system in the
neurochemistry of schizophrenia (see section 1.4.2). Administration of LSD to
rats is known to cause PPI deficits (Geyer et al., 1987); also, it has been
demonstrated that both LSD and mescaline can alter glutamatergic
neurotransmission in rats (Yamada et al., 1999). However, despite the
acknowledged involvement of the serotoninergic system in schizophrenia, the
usefulness of the administration of 5-HT₂A agonists as a model of the disease is
controversial. In fact, chronic administration of LSD induces behavioural
habitation, which is not compatible with schizophrenia (Marcotte et al., 2001)
and other pharmacological models can induce a broader range of schizophrenia-
like neurochemical and behavioural alterations (5.1.3).
1.4.4.3 Glutamatergic transmission inhibitors

The psychotomimetic effect of NMDA antagonists is perhaps closer to schizophrenia than any other psychoactive compound. Unlike amphetamine and LSD, phencyclidine-induced psychosis can mimic both the positive and negative symptoms and cognitive deficits in humans, therefore the glutamatergic model promises to be more comprehensive than the dopaminergic one (reviewed by Jentsch et al., 1999). Indeed, phencyclidine (PCP) models are widely used, and their face validity is demonstrated by several behavioural tests. For details on PCP pharmacology and PCP models of schizophrenia refer to section 5.1.

1.4.5. Genetic models of schizophrenia.

Another strategy to reproduce schizophrenic symptoms in animals is genome manipulation. It is clear that genes play a key role in the aetiology of this disease (see section 1.6), therefore genetic animal models promise new insights. Knockout mutants are the most common genetic models. As schizophrenia is a complex polygenic disease, it is unlikely that the deletion of a single gene can reproduce comprehensively the features of the human disease. Nevertheless, some genes are known to be very influential. It would be interesting to understand the behavioural and biological consequences of their deletion, and how they interact with other relevant genes.

Consistent with the dopamine hypothesis and the glutamate hypothesis, the disruption of key genes for neurotransmission such as the dopamine transporter and the NR-1 subunit of the NMDA receptor, causes schizophrenia-like biological and behavioural changes (see below). The dopamine transporter knockout (DAT-KO) mutant mice show hyperlocomotion and increased stereotypic behaviours (Ralph et al., 2001; Spielewoy et al., 2000), as well as deficits in sensorimotor
gating (Ralph et al., 2001). Neuroleptic treatment reverses the hyperlocomotion, showing that this model has a degree of predictive validity. The NR-1 knockdown (NR1-KD) mice also show increased locomotion and stereotypy; in addition, social interaction is consistently impaired (Mohn et al., 1999). Typical antipsychotics reverse the hyperlocomotion, while clozapine ameliorates the impairments in social behaviour (Mohn et al., 1999).

The deletion of some genes abnormally expressed in schizophrenia can provoke histological and cytoarchitectural abnormalities similar to those observed in schizophrenia. The reeler mouse, which has the Rei1 gene mutated, is affected by abnormal neuronal migration and growth during development (Curran et al., 1998). Mutation of the Dabl gene causes similar cytoarchitectural abnormalities (Rice et al., 1998), suggesting that these two genes are involved in neurodevelopmental processes possibly disrupted in schizophrenia.

Although these genetic models are far from mimicking all the symptomatic features of schizophrenia, they can be useful to highlight the consequences of specific alterations in the neurotransmission pathways. The advances in linkage and gene expression studies could pave the way to the development of new, relevant genetic models. As many genes are involved in schizophrenia, it would be as useful, as well as challenging, to create animals with a complex genotype producing a schizophrenia-like phenotype. In this way a degree of construct validity could be achieved.
1.5 Schizophrenia and genes

If the neuropathology of schizophrenia needs to be clarified, the aetiology of this disease is even more obscure. Although a definitive cause of schizophrenia is far from being discovered, it is widely accepted that genetic factors play a very important role. The first evidence comes from the epidemiological studies performed during decades of research. An invaluable analysis of epidemiological studies from different countries, leading to interesting conclusions was performed by Gottesman (1991).

The average risk for developing schizophrenia is much higher in schizophrenics' close relatives than in the general population (Gottesman, 1991). Furthermore, the higher the genetic correlation with a patient, the higher the risk of developing the illness. For instance, monozygotic twins of schizophrenics, who share the same genome, have an average risk of 50%; first-degree relatives such as patients' siblings (including fraternal twins) or children have an average risk of about 15%; while the risk associated to the general population is 1% (Gottesman, 1991). Interestingly, adoption studies show that the average risk does not change when children are brought up by healthy adoptive parents rather than by their natural, schizophrenic parents, suggesting that genetic factors are more influential than sharing the same stressful environment of a schizophrenic parent (Gottesman, 1991). This finding is confirmed by the fact that spouses of patients have the same average risk as the general population. Although epidemiological data seem to support a genetic model of the disease, the pattern of familial risk demonstrates that schizophrenia is not caused by a single gene mutation inherited according to the simple Mendelian model; but it is rather due to a multifactorial-polygenic condition probably combined with environmental factors.
1.5.1. Is schizophrenia correlated with specific gene alterations?

Numerous attempts to identify schizophrenia-susceptibility genes by linkage and association studies have led to interesting but often contradictory results (see chapter 6). The complexity of the neuropathology of schizophrenia and the heterogeneity of the schizophreniform phenotypes contribute to the difficulties encountered by the researchers exploring the genetics of this disorder. However, the genetic approach may have the advantage of identifying genes that are necessarily involved in the aetiology of the disease, rather than being secondary alterations caused by pathological processes or pharmacological treatments (Ross et al., 2006).

Although the results of studies based on a genetic approach remain difficult to interpret due to the many inconsistencies, recent meta-analysis have contributed to clarify the useful information reported in the single studies. (see chapter 6) (Badner et al., 2002; Lewis et al., 2003; Harrison et al., 2005). Interestingly, the data available from the literature convincingly shows that schizophrenia is linked to some genes that are possibly involved in the neurochemical changes observed in this disease.

Straub et al. (2002) first showed that the gene encoding for dysbindin is significantly linked to schizophrenia. This finding has been replicated in subsequent studies (Ross et al., 2006) Dysbindin is localised in the presynaptic terminals and may have a role in signal transduction.

Neuregulin-1 is a strong candidate gene for schizophrenia (Harrison et al., 2006; Stefansson et al., 2003; Stefansson et al., 2002); neuregulin-1 is present in glutamatergic synapses and has an important role in neurodevelopment as it is involved in neuron migration and cellular differentiation. The involvement of neuregulin-1 in the neuropathology of schizophrenia is one of the most consistent findings from genetic studies and is supported by gene expression data (Law et al., 2006).
The COMT (Catechol-O-methyltransferase) gene has also been shown to be associated to schizophrenia (Kunugi et al., 1997; Li et al., 2000; Shifman et al., 2002). This gene encodes for the enzyme responsible for the clearance of dopamine from the synaptic terminal; therefore it could be directly involved in the neurochemical mechanisms underlying the neuropathology of schizophrenia (Craddock et al., 2006; Tunbridge et al., 2006).

Another interesting finding is the disruption of the gene DISC1 (Disrupted in schizophrenia) due to a chromosomal translocation identified in a Scottish population affected by schizophrenia, schizoaffective disorder and major depression (Blackwood et al., 2001; Millar et al., 2001). Although the exact function of DISC1 is unclear, this gene could be involved in brain development, synaptic transmission and plasticity (Ross et al., 2006). Interestingly, the study of the Scottish phenotype using imaging and neuropsychology techniques suggests that DISC1 could be particularly relevant to cognitive endophenotypes, as it is associated with altered hippocampal function and altered working memory in this group of patients (Callicott et al., 2005; Cannon et al., 2005; Porteous et al., 2006).

The findings briefly summarised above are among the most consistent data available to date from genetic studies on schizophrenia (see also chapter 6). This type of approach has generated a vast amount of useful data whose discussion is beyond the scope of this thesis. Many of these results have often proved to be impossible to reproduce, even though the inconsistency of data from genetic studies should not induce excessive scepticism. In fact, variability may reflect the fact that schizophrenia is a heterogeneous polygenic disorder. Nevertheless, in this scenario it is clear that the identification of susceptibility genes and, above all, their interaction with other genes and environmental factors cannot be found by genome-wide scans alone, but requires biological evidence from studies analysing their impact on the neuropathology of schizophrenia at different levels (Harrison et al., 2005).
1.5.2. Microarray analysis of gene expression in schizophrenia

Gene expression studies have given an important contribution to the understanding of molecular pathology in psychiatric disorders (reviewed by Iwamoto et al., 2006; Mimics et al., 2006). This approach is very useful to complement the information from genetic studies. In fact, expression changes in human post mortem brain tissue can be due not only to genetic abnormality, but also to environmental factors and secondary indirect causes. In a complex condition such as schizophrenia, the neuropathological phenotype results from a combination of primary abnormalities and secondary changes (Mimics et al., 2006). The primary alterations can be a direct consequence of genetic abnormality or can have environmental origin. These changes are likely to cause a number of secondary downstream alterations that can contribute crucially to the pathological mechanisms (Mimics et al., 2006). As an example, adapitional changes can be considered as a subset of secondary alterations reflecting the attempt of the cells to re-establish physiological conditions in response to a primary lesion. Current knowledge suggests that secondary gene expression changes in the schizophrenic brain greatly outnumber those that are directly caused by polymorphisms in the encoding gene (Mimics et al., 2006). Importantly, secondary alterations can be very informative, as they can underlie similar pathological processes in patients that do not show the same genetic abnormalities.

One of the advantages of gene expression studies over genome-wide scans is that the former strategy also allows the analysis of pathological changes caused by environmental and secondary factors. However, traditional approaches that allow the expression analysis of one or few genes at a time can be severely limiting in schizophrenia research for a number of reasons. Firstly, the neuropathology of this disease is likely to be due to small expression changes of multiple genes; secondly, the limited knowledge about the etiology of schizophrenia complicates the formulation of hypotheses and the choice of specific candidate genes.
In this context, the recently developed technologies for transcriptome analysis based on oligonucleotides microarray can be very helpful, as they allow genome-wide scans at the mRNA level in a comprehensive and hypothesis-free manner (see also chapter 3). Microarray analysis has been recently employed for the transcriptome profiling of a number of psychiatric conditions such as autism, anxiety, major depression, bipolar disorder and schizophrenia (reviewed by Mirmics et al., 2006). Not surprisingly, some of the data on gene expression in schizophrenic post mortem brain tissue are contradictory probably due to common issues such as variability of diagnostic criteria and comorbidity with other disorders. Nevertheless, this powerful technology has allowed the identification of some interesting expression changes of multiple genes belonging to functional classes controlling synaptic transmission, energy metabolism and functions of non-neuronal cell populations such as oligodendrocytes (Iwamoto et al., 2006). Moreover, a number of findings from microarray studies are concordant with previous data showing expression changes of genes controlling the GABAergic and glutamatergic neurotransmission systems in schizophrenia both at the mRNA and protein level (Mirmics et al., 2006).

One of the most consistent finding from expression studies in schizophrenia is the decreased expression of synaptic markers. Interesting data from microarray studies demonstrate that the mRNAs encoding for proteins that participate in synaptic function, such as synaptophysin, synaptotagmin I, synaptotagmin IV and synaptic plasma membrane proteins (SNAP 23 and SNAP 25) are underexpressed in the schizophrenic prefrontal cortex (Mirmics et al., 2000; Hemby et al., 2002). Mirmics et al., (2000) also showed that the expression changes reported in their microarray study are not likely to be a consequence of pharmacological treatment with neuroleptics, as rhesus monkeys chronically treated with haloperidol did not show those alterations.

Interestingly, the expression of genes controlling the mitochondrial energy metabolism also seems to be altered in schizophrenia. Middelton et al. (2002) showed that transcripts encoding for proteins involved in ornithine and polyamine metabolism, mitochondrial malate shuttle system, transcarboxylic acid cycle,
aspartate and alanine metabolism, and ubiquitin metabolism are underexpressed in the schizophrenic prefrontal cortex. Iwamoto et al. (2005) also reported downregulated expression of genes controlling the mitochondrial metabolism in schizophrenia, although this abnormality was not specific as it was also found in a group of bipolar disorder patients. Another study that combines data from transcriptomics and proteomics approaches conclude that altered expression of genes controlling mitochondrial metabolism and oxidative stress can be a landmark of the expression profile of schizophrenia (Prabakaran et al., 2004). Although the abnormalities summarised above may be present in other psychiatric conditions (Iwamoto et al., 2005) and are not likely to be primary deficits, these convincing findings show how microarray-based transcriptome analysis can contribute to the identification of important characteristics of schizophrenia that are not directly related to the neurochemical and neurotransmission alterations.

Recent microarray studies support previous evidence suggesting that white matter changes are a feature of schizophrenia (reviewed by Davis et al., 2003). Evidence of downregulation of oligodendrocyte transcripts in schizophrenia first came from the microarray study performed by Hakak et al. (2001). This group reported that 35 genes controlling myelination and oligodendrocyte function are underexpressed in the prefrontal cortex of elderly, hospitalised patients. Another microarray study also reported underexpression of oligodendrocytic transcripts in a younger group of patients (Pongrac et al., 2002), suggesting that this type of molecular abnormality is not found in chronic, elderly subjects only. Further evidence of gene expression abnormalities in the oligodendrocytes of schizophrenic brains was reported by Tkachev et al., (2003) and Sugai et al. (2004), suggesting that dysregulation of white matter function and development could be a core feature of schizophrenia neuropathology.

Data from transcriptome profiling of schizophrenic post mortem brain tissue also contributed to confirm precedent evidence of altered glutamatergic and GABAergic neurotransmission systems (Mimics et al., 2006). One of the best-documented molecular abnormalities in the schizophrenic prefrontal cortex is the altered function of the chandelier subset of GABAergic interneurons, which show
specific changes including decreased density of parvalbumin-immunoreactive axon cartridges (Lewis et al., 2000) and downregulation of genes controlling the biosynthesis of GABA, such as GAD67 (Akbarian et al., 1995; Lewis et al., 2000) (see also section 1.4.4). Interestingly, the downregulation of GAD67 in the schizophrenic prefrontal cortex was also found in the microarray study performed by Mirmics et al. (2000). The same study also reported expression changes of the glutamatergic system, which showed underexpression of the mRNA encoding for the AMPA2 receptor.

Although microarray technology has been applied to schizophrenia research only recently, it has already given a substantial contribution to the understanding of molecular alterations underlying this psychiatric disease. It is predictable that in the near future transcriptomic profiling will produce even more robust and reliable data, following technological improvements of the array chips. In fact, the current literature reports a mixture of consistent data, such as the findings summarised above, but also less reliable results that could not be reproduced independently (Mirmics et al., 2006; Iwamoto et al., 2006). This inconsistency is partially due to the variability observed when different microarray platforms are employed for transcriptome profiling. However, the new generation of oligonucleotide chips is already setting higher standards of reliability (see also section 3.1.4).

On the other hand, it is important to emphasise that the challenge of understanding the molecular abnormalities that underlie schizophrenia cannot be resolved by technological advances alone, as inconsistency of data from post mortem human tissue also depend on a number of other factors. Effect of chronic pharmacological treatment, diagnostic inconsistency, comorbidity of other diseases especially in older patients, and diversity between subjects in crucial parameters such as age, drug abuse and post mortem interval are all confounding factors that are very difficult, if not impossible to eliminate when analysing human brain tissue (Pongrac et al., 2002; Mirmics et al., 2004). Obviously, despite these issues, transcriptomic profiling of human diseased tissue remains one of the strongest methodologies to elucidate the molecular basis of schizophrenia, especially if the findings are supported by evidence from studies based on other
approaches. In this context animal models can be an invaluable source of material to study the molecular basis of specific schizophrenia-like neurobiological and behavioural alterations under uniform and rigorously controlled conditions (refer to sections 1.4 and 5.1).

Another source of variability in gene expression studies of psychiatric disorders is the neuroanatomical and cellular diversity of the brain areas that are involved in the neuropathological processes. This issue is particularly relevant, because modest changes affecting specific subregions or even cell populations are likely to have a great impact on the neurobiological processes, but they are difficult to detect when the samples include material from non-relevant neighbouring regions. This complication can be potentially overcome using advanced methods of sample collection, such as laser-assisted microdissection, that allow to perform transcriptome profiling of specific brain subregions or even cell populations (refer to sections 3.1.4 and 3.1.5).

Despite the enormous efforts to clarify the aetiology and the neuropathological basis of schizophrenia, many aspects of this disorder remain unclear. However, the growing evidence from studies based on different approaches is shedding light on important molecular abnormalities that lie at the basis of the complex symptomatology shown by the patients (Harrison et al., 2005; Ross et al., 2006). It cannot be overemphasised that progress in the understanding of schizophrenia molecular pathology can only come from coordinated efforts and integration of data obtained using different strategies. While genetic studies and gene expression of post mortem brain tissue remain crucial to investigate the molecular alterations associated with the human disease, information obtained from animal models is an essential integration and is invaluable for the analysis of specific neurobiological and behavioural abnormalities (see section 1.4). Identification of novel potential drug targets and drug development will also benefit from a better
understanding of the schizophrenia-like molecular abnormalities observed in animal models.
2. Materials and Methods
2.1 Materials

Phencyclidine hydrochloride (PCP) was obtained from Sigma-Aldrich, Poole, UK.

Diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), polyadenylic acid, isopentane and poly-L-lysine were all obtained from Sigma-Aldrich, Poole, UK. Acetic acid, Amberlite IRN-150L monobed mixed resin, sodium chloride (NaCl) and sodium pyrophosphate were all obtained from BDH, Poole, UK. Ethylenediaminetetra-acetic acid (EDTA), methanol, sodium dihydrogen orthophosphate (NaH$_2$PO$_4$), di-sodium hydrogen orthophosphate (Na$_2$HPO$_4$) were all obtained from Fisher Chemicals, Loughborough, UK. Dextran sulphate was obtained from Amersham Biosciences, Chalfont St Giles, UK. 0.9% saline was obtained from Baxter Healthcare Ltd, Northampton, UK.

The source of other materials used for histology, immunohistochemistry, molecular biology and in situ hybridisation techniques is reported in brackets within the description of the relevant procedures below.
2.2 Laser-assisted microdissection/microarray study

2.2.1 Animals and tissue preparation

Male hooded Long Evans rats (Harlan, Oxon, UK) were housed under a 12h light/dark cycle, 55% humidity and received standard rat chow and water ad libitum. The animals, weighing 290-330g, were killed by cervical dislocation, then decapitated, the brains removed and quickly frozen in isopentane at -42°C. The brains were stored at -80°C until further processing. Experimental procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986 and associated guidelines.

Six rat brains were used to cut 10μm coronal sections in a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany) at -20°C from the following Bregma levels according to Paxinos and Watson (1998): 3.7mm, prefrontal cortex and -1.4mm, anterior thalamus. The sections were mounted onto PET membrane slides (Leica Microsystems), previously treated with RnaseZap (Ambion, Austin, TX, USA) for nuclease inactivation. Briefly, the slides were incubated in undiluted RnaseZap solution for 5 minutes, and then rinsed with diethylpyrocarbonate (DEPC)-treated water.

The DEPC-treated water was prepared by adding 0.1% DEPC (Sigma) to deionised water followed by overnight incubation and autoclaving at 120°C. This treatment was used to ensure the deactivation of nucleases present in the deionised water.

After mounting the sections, the slides were immediately frozen on dry ice, avoiding air-drying to preserve morphological integrity. The slides were stored at -80°C in a box containing silica gel until further processing.
Figure 2.1
Flow-chart summarising the steps of the Laser-assisted Microdissection/Microarray experimental strategy for gene expression profiling of the rat Prelimbic cortex (PrL), Ventral Orbital cortex (VO), Primary Motor cortex (M1), and Reticular Thalamic nucleus (Rt). Refer to sections indicated in brackets for detailed description of each method.
2.2.2 Histological staining

All the steps of the histological staining procedure were performed under rigorous nuclease-free conditions. The Coplin jars used for the fixing and washing solutions were baked at 180°C for a minimum of 2 hours to ensure nuclease deactivation. The slides were taken from -80°C and immediately fixed in ice-cold acetone for 3 minutes. After fixation, the slides were placed horizontally on a rack; the staining solution was pipetted onto the sections and allowed to incubate for 3 minutes at room temperature. The staining solution consisted of 1% toluidine blue (Sigma) dissolved in DEPC-treated water. The solution was filtered using 0.2 μm pore size filters (Minisart, Sartorius, Epsom, UK) before use. After staining, the slides were washed twice in DEPC-treated water for 15 seconds and once in 75% ethanol for 1 minute at room temperature. The stained slides were air-dried for 3-5 minutes, stored in a box with silica gel and immediately used for the microdissection.

2.2.3 Immunohistochemical staining

Sections adjacent to those used for LMD (10 μm, anterior thalamus level) were mounted on poly-L-lysine coated glass slides (previously baked at 180°C and coated in a 0.01% poly-L-lysine solution made up in DEPC-treated water). The slides were immediately frozen on dry ice until further processing.

Frozen sections were fixed in ice-cold acetone for 3 minutes, then rinsed in phosphate buffered saline (PBS: 1.3M NaCl, 70mM Na₂HPO₄ and 30mM NAH₂PO₄ in DEPC-treated water). The slides were transferred in a H₂O₂ solution (0.3 % v/v H₂O₂ in methanol) for 10 minutes for quenching of endogenous peroxidase. Sections were incubated with normal goat serum (5% diluted in PBS,
Vector Laboratories, Peterborough, UK) for 5 minutes, followed by incubation with anti-GAD65/67 primary antibody (rabbit anti-glutamic acid decarboxylase 65/67, IgG fraction, Sigma; 1:1000 dilution in PBS) for 10 minutes. Sections were washed with a stream of PBS, and then incubated with secondary antibody (biotinilated anti-rabbit IgG raised in goat, Vector Laboratories, 1:500 dilution in PBS with 3% normal goat serum) for 5 minutes. Sections were washed with a stream of PBS, then incubated with avidinDH-biotinilated horseradish peroxidase complex (ABC reagent, Vector Laboratories) for 3 minutes. Sections were washed with a stream of PBS, then incubated with nickel chloride-3,3'-diaminobenzidine (DAB substrate kit, Vector Laboratories) for 2 minutes followed by a wash in deionised water and air-drying. The dry, stained sections were coverslipped using Histomount™ histological mounting medium (National Diagnostics, UK).

2.2.4 Identification of regions and Laser Microdissection

The prelimbic cortex (PrL), the ventral orbital cortex (VO), and the primary motor cortex (MI) were identified on the toluidine-blue stained sections referring to the anatomical landmarks reported by Paxinos and Watson (1998). The reticular thalamic nucleus (Rt) was also identified referring to Paxinos and Watson (1998). Additionally, the exact position of the Rt in the toluidine blue stained section was clarified by comparing it to the adjacent immuno-stained section (see above). As the Rt is very rich in GABAergic neurones, GAD65/67 immunoreactivity can be considered a marker for this anatomical region.

Microdissections were performed using the Leica AS LMD system (Leica Microsystems, Wetzlar, Germany).

The microscope stage and the rack holding the tubes for collection of the microdissected samples were cleaned using tissue soaked in RNase Zap (Ambion, Austin, TX, USA) to prevent nuclease contamination of the sections.
The dehydrated stained sections were placed on the microscope stage, and the UV laser was used to dissect the region of interest. The procedure was performed according to a conservative estimate of the anatomical boundaries to prevent contamination of the sample with material from neighbouring regions. All the microdissections were performed using the following laser parameters: aperture 14; intensity 45; speed 2; offset 23 using 4X objective. The microdissected tissue was collected by gravity in a sterile tube cap placed underneath the slides containing 20 μl of RLT lysis buffer (RNeasy Micro kit, Qiagen, Valencia, CA, USA). This method ensured that the tissue would be dissolved in a nuclease-deactivating buffer immediately after dissection. After adjusting the volume to 75 μl with RLT buffer and spinning to collect the sample at the bottom of the tube, the crude lysates were frozen on dry ice. The time elapsed between the staining procedure and the collection of the microdissected sample in RLT buffer was kept to a minimum (30 minutes maximum) to prevent RNA degradation. For the same reason, each section was used for the microdissection of one sample only. Each of the 24 samples (four brain regions from six biological replicates) consisted of one specific region dissected bilaterally from one stained section. The crude lysates were stored at -80°C until RNA isolation.

2.2.5 Total RNA isolation

Total RNA was isolated using the RNeasy Micro kit (Qiagen, Valencia, CA, USA). The frozen samples were thawed at room temperature and vortexed for 30 seconds. 75 μl of 70% ethanol were added to the homogenised lysates. After mixing by pipetting, the samples were loaded onto RNeasy MinElute Spin Columns (RNeasy Micro kit, Qiagen, Valencia, CA, USA) and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and the columns were washed according to the following procedure: 700 μl buffer RW1 (RNeasy Micro
kit, Qiagen, Valencia, CA, USA) followed by 15 seconds centrifugation at 10,000 rpm; 500 μl buffer RPE (RNeasy Micro kit, Qiagen, Valencia, CA, USA) followed by 15 seconds centrifugation at 10,000 rpm; 500 μl 80% ethanol followed by 2 minutes centrifugation at 10,000 rpm. The columns were transferred into a collection tube and eluted with 13 μl RNase-free water (Ambion Austin, TX, USA) by centrifuging for 1 minute at 13,000 rpm. The volume of eluate collected was approximately 11 μl. An aliquot of the total RNA solution (2.4 μl) was used for quality assessment performed on the Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip kit (Agilent Technologies, Palo Alto, CA, USA). The samples were stored at -80°C until further processing.

2.2.6 RNA amplification

In order to obtain enough material for the microarray hybridisation, two rounds of linear amplification were performed. The total RNA was linearly amplified using the ExpressArt mRNA Amplification kit, Nano Version (Artus-Biotech, Hamburg, Germany). This kit employs a technology that is a variation of the classic linear isothermal amplification strategy based on in vitro transcription with T7 RNA-polymerase (Van Gelder et al., 1990; Kiberwine et al., 1992). Briefly, the original mRNA is reverse-transcribed to cDNA using oligo(dT)s as primers, without introducing the T7 promoter. In the following step, double stranded cDNA (dsDNA) is generated using a special “Trinucleotide Primer” (Box-random-trinucleotide primer), that allows preferential priming near the 3’-ends of nucleic acid molecules. At this stage the functional T7 promoter is inserted by denaturing the dsDNA, and priming the second strand in reverse orientation, using a T7-promoter/oligo(dT). The product of this reaction is a dsDNA with a functional T7-promoter at one end and the Box sequence tag at the other end. This product can be used as template for the in vitro transcription.
Figure 2.2

Flow-chart summarising the steps of the two rounds of RNA linear amplification performed in order to obtain enough material for the microarray hybridisation from each individual microdissected sample. Refer to sections indicated in brackets for detailed description of each step.
generating amplified antisense RNA (aRNA) with defined sequences at both ends. The aRNA can be re-amplified without further shortening of the fragments, as the reverse transcription is primed using the Box sequence tag instead of random primers. After the second strand synthesis using T7-promoter/oligo(dT) primers, the dsDNA can be used as template for the second round of *in vitro* transcription, incorporating biotin-labelled ribonucleotides.

The detailed procedure describing the two-rounds amplification of each sample is reported below (see figure 2.2). All reagents were provided with the ExpressArt mRNA Amplification kit, Nano Version (Artus-biotech, Hamburg, Germany), except for the materials used for the aRNA purification. This procedure was performed using the RNaseasy Mini kit (Qiagen, Valencia, CA, USA). The manufacturers do not specify the concentrations of the compounds in the buffers and reagents solutions.

No-template negative control amplifications were carried out in parallel with samples. Positive control amplifications to check the performance of the kit were also performed using total RNA provided by the manufacturer.

### 2.2.6.1 First round - First strand cDNA synthesis

The total RNA eluates were thawed at room temperature. 1.0 µl dNTP-Mix and 1.5 µl oligo(dT) were added to 7.5 µl total RNA solution (7.5 µl RNase-free water for the no-template negative controls) and incubated for 4 minutes at 65°C in a thermocycler with heated lid (PCR Sprint, Thermohybrid, Waltham, MA, USA), followed by cooling at 37°C. A mix composed of DEPC-treated water (4 µl), 5X reverse transcription buffer (4µl), RNase inhibitor (1µl), and reverse transcriptase enzyme (1µl) was added to the reaction tube. The reaction mix was incubated in a thermocycler using the following conditions: 37°C for 5 minutes, 42°C for 50 minutes, 45°C for 10 minutes, 50°C for 10 minutes and 70°C for 15 minutes. After incubation the samples were immediately cooled on ice.
2.2.6.2 First round - RNA removal

The RNA was removed from the hybrid double stranded product by adding a mix composed of DEPC-treated water (3 μl), 5X extender buffer (1μl) and RNase (1μl). The reaction mix was incubated for 20 minutes at 37°C in a thermocycler.

2.2.6.3 First round - Second strand cDNA synthesis

The following mix was added to the reaction tube: DEPC-treated water (14 μl), 5X extender buffer (4μl), "Trinucleotide Primer" (1μl), and d-NTP-mix (1μl). The reaction mix was incubated in a thermocycler at 96°C for 1 minute followed by cooling at 37°C for 1 minute to allow primers annealing. A mix composed of DEPC-treated water (3 μl), 5X Extender Buffer (1μl), and Extender Enzyme A (DNA polymerase, 1 μl) was added to the reaction tube, followed by incubation at 37°C for 30 minutes in a thermocycler. At this stage the product consisted of dsDNA with the Box sequence tag incorporated in one end. The following steps are designed to incorporate the functional T7-promoter in the dsDNA. The following mix was added to the reaction tube: DEPC-treated water (3 μl), 5X Extender Buffer (1μl), and Primer Erase enzyme (1 μl). The reaction mix was incubated at 37°C for 5 minutes and 96°C for 6 minutes in a thermocycler. The tube was immediately placed on ice. After spinning to collect the liquid, 5 μl of T7-promoter/oligo(dT) primer were added to the reaction mix, followed by incubation in a thermocycler at 96°C for 1 minute and 37°C for 1 minute to allow primers annealing. A mix composed of DEPC-treated water (2 μl), 5X extender buffer (2μl), and extender enzyme B (DNA polymerase, 1 μl) was added to the reaction tube, followed by incubation at 37°C for 30 minutes and at 65°C for 15 minutes in a thermocycler. The reaction mix was cooled on ice.
2.2.6.4 First round — *cDNA purification and concentration*

The dsDNA was purified using spin columns (supplied with the ExpressArt mRNA Amplification kit) and then concentrated by ethanol precipitation. After synthesis, the dsDNA reaction mix was diluted with 350 μl Binding Buffer, and 3 μl Carrier DNA were added. The solution was loaded onto the spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the column washed twice with 500 μl Washing Buffer, centrifuging at 13,000 rpm for 1 minute. The dsDNA was eluted with 50 μl Elution Buffer, incubating for 1 minute at room temperature and centrifuging at 13,000 rpm for 1 minute. The elution step was repeated to maximise the amount of dsDNA extracted from the column. The total volume of eluate collected was 100 μl. The dsDNA was concentrated by ethanol precipitation. 10 μl Sodium Acetate (3M solution) and 2 μl Precipitation Carrier (Pellet Paint Co-Precipitant, Merck Biosciences Ltd, UK) was added to the eluate. After mixing, adding 220 μl absolute ethanol, and incubating for 2 minutes at room temperature, the mix was centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet washed with 200 μl 70% ethanol. The sample was centrifuged at 13,000 rpm for 5 minutes at room temperature, and the supernatant completely removed. The pellet was air-dried leaving the reaction tube open for 5 minutes at room temperature in a sterile hood to prevent air-borne contamination. The dsDNA pellet was dissolved in 8 μl Solubilisation Buffer.

2.2.6.5 First round — *in vitro transcription*

The dsDNA incorporating a functional T7 promoter was used as template for the amplification via *in vitro* transcription (IVT). A mix composed of NTP mix (8
μl), 10X IVT Buffer (2μl), and T7 RNA polymerase (2μl) was added to the dsDNA solution from the purification/concentration step. The IVT reaction mix was incubated overnight at 37°C in a hybridisation oven (Shake’n’Stack, ThermoHybaid, Waltham, MA, USA).

2.2.6.6 First round – aRNA purification and concentration

After overnight incubation, 1 μl DNase I was added to the IVT reaction mix, followed by incubation at 37°C in a thermocycler. Once the DNA template was degraded, the amplified aRNA was purified using the RNeasy Micro kit (Qiagen, Valencia, CA, USA). The aRNA solution was diluted with 80 μl RNase-free water, then 350 μl RLT Buffer were added. After mixing, 250 μl absolute ethanol was added to the solution, which was then loaded onto the spin column. The sample was centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and the column washed with 500 μl RPE Buffer, centrifuging as for the previous step. The washing step was repeated with the same amount of buffer, centrifuging at 13,600 rpm for 2 minutes. The aRNA was eluted with 50 μl RNase-free water, incubating for 1 minute at room temperature and centrifuging at 13,000 rpm for 1 minute. The elution step was repeated to maximise the amount of aRNA retrieved from the column. The final elution volume was 100 μl. The aRNA was then concentrated by ethanol precipitation. The procedure and the reagents are the same employed for the dsDNA concentration (see above). The aRNA pellet was dissolved in 5 μl RNase free water.

2.2.6.7 Second round – First strand cDNA synthesis

All the aRNA synthesised in the first round of amplification was used as template for the second round reverse transcription. A mix composed of dNTP-
mix (1 μl), Box primer (2 μl) and Reaction Additive (2 μl) was added to the 5 μl of aRNA solution from the concentration step. The reaction mix was incubated at 65°C for 4 minutes in a thermocycler, then immediately cooled at 45°C. A mix composed of RNase free water (4 μl), 5X Reverse Transcription Buffer (4 μl), RNase inhibitor (1 μl) and Reverse Transcriptase enzyme (1 μl) was added to the reaction mix, followed by incubation at 45°C for 30 minutes and 70°C for 15 minutes. After incubation, the sample was immediately cooled on ice.

2.2.6.8 Second round - RNA removal

This step was performed as described for the first round of amplification.

2.2.6.9 Second round - Second strand cDNA synthesis

A mix composed of RNase free water (10 μl), 5X Extender Buffer (4 μl), T7-promoter/oligo(dT) primer (5 μl) and dNTP-mix (1 μl) was added to the reaction mix, followed by incubation at 96°C for 1 minute and 37°C for 1 minute to allow the primers to anneal. A mix composed of RNase free water (3 μl), 5X Extender Buffer (1 μl) and Extender Enzyme B (DNA polymerase, 1 μl) was added to the reaction mix and incubated at 37°C for 30 minutes and 65°C for 15 minutes in a thermocycler. After incubation, the sample was immediately cooled on ice.

2.2.6.10 Second round - cDNA purification and concentration

The reaction solution was diluted with 275 μl Binding Buffer and 3 μl Carrier DNA was added. This mixture was loaded onto a spin column. The rest of the procedures of purification and concentration were performed as described for the
first round of amplification. The dsDNA pellet was dissolved in 8 µl Solubilisation Buffer and frozen at -80°C until further processing.

2.2.7 Array target preparation and purification

The dsDNA synthesized in the second round of linear amplification was used as template for the labelling via \textit{in vitro} transcription. The labelling IVT is in fact the last step of the amplification procedure; at the same time the product of this reaction is the biotin-labelled aRNA needed for the microarray hybridisation. The labelling IVT is essentially an ordinary \textit{in vitro} transcription reaction performed using biotin-labelled ribonucleotides. The labelling IVT was performed using the GeneChip IVT Labelling Kit (Affymetrix, Santa Clara, USA). All reagents were provided with the GeneChip IVT Labelling Kit. The purification of the labelled aRNA was performed using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). All the buffers used for the purification were supplied with this kit. The manufacturers do not specify the concentrations of the compounds in the buffers and reagents solutions. The labelling IVT and purification steps were performed for no-template negative controls in parallel with the samples.

4 µl dsDNA from the second round of amplification (50% of the total yield) was added to a mix composed of 10X IVT Labelling Buffer (2 µl), IVT Labelling NTP mix (6 µl), IVT Labelling Enzyme (2 µl), RNase-free water (6 µl). The reaction mix was incubated at 37°C for 16 hours in a thermocycler.

The labelled aRNA was purified according to the same procedure described for the purification of the first round IVT product (see above). The labelled aRNA was eluted in 30 µl RNase-free water. Aliquots of the eluate from the samples and the negative controls were used for the spectrophotometric quantification (NanoDrop, Wilmington, USA) and the quality assessment (Agilent 2100
Bioanalyzer, using the RNA 6000 Pico LabChip kit; Agilent Technologies, Palo Alto, CA, USA).

**2.2.8 Labelled aRNA fragmentation**

The labelled aRNA must be fragmented prior to microarray hybridisation to obtain optimal assay sensitivity. The Fragmentation Buffer (Affymetrix, Santa Clara, USA) breaks down the full-length aRNA to 35 to 200 base fragments by metal-induced hydrolysis.

A volume of eluate from the previous step containing 15 μg aRNA was added to 6 μl Fragmentation Buffer. The fragmentation mix volume was adjusted to 30 μl with RNase-free water. The mix was incubated at 94°C for 35 minutes, then immediately cooled on ice. Fragmented labelled aRNA was stored at -20°C until hybridisation.

**2.2.9 Microarray Hybridisation**

24 Rat Genome 230 2.0 GeneChip arrays (Affymetrix, Santa Clara, USA) were used for this study, each hybridising a sample derived from the four brain regions of six biological replicates. This array platform allows the analysis of 30,000 transcripts and variants from over 28,000 well-substantiated rat genes.

The hybridisation cocktail was prepared with the following components: 15 μg fragmented labelled aRNA (the entire product of the fragmentation reaction described above); 5 μl Control Oligonucleotide B2 (3nM, Affymetrix, Santa
Clara, USA); 15 µl 20X Eukaryotic Hybridisation Controls bioB, bioC, bioD, cre (Affymetrix, Santa Clara, USA); 3 µl herring sperm DNA (10 mg/ml solution, Promega, Madison, USA); 3 µl bovine serum albumin (50 mg/ml solution, Invitrogen Life Technologies, Carlsbad, USA); 150 µl 2X hybridisation buffer; 30 µl DMSO (Sigma-Aldrich); the volume was adjusted to 300 µl with RNase-free water. The 2X hybridisation buffer was prepared with the following components: 8.3 mL 12X MES Stock Buffer (1.22M MES, 0.89M [Na⁺]; MES hydrate and MES Sodium Salt were purchased from Sigma-Aldrich), 17.7 mL RNase-free 5M NaCl solution (Ambion, Austin, USA), 4.0 mL EDTA (0.5M EDTA Disodium Salt solution, Sigma-Aldrich), 0.1 mL 10% Tween-20 (Pierce, Rockford, USA), 19.9 mL DEPC-treated water. The hybridisation cocktail was heated at 99°C for 5 minutes, and then cooled at 45°C for 5 minutes in a heat block.

Meanwhile, the arrays were equilibrated at room temperature and filled with 1X hybridisation buffer, followed by incubation at 45°C for 10 minutes with rotation in the hybridisation oven (Affymetrix, Santa Clara, USA). This step was performed to ensure that the arrays would be at the right temperature and hydration state for the hybridisation procedure. After incubation, the buffer was removed and discarded.

The hybridisation cocktail was centrifuged at 13,000 rpm for 5 minutes to remove all the insoluble material. 200 µl of the clarified hybridisation mixture was loaded onto the array, followed by incubation at 45°C for 16 hours with rotation at 60 rpm.

2.2.10 Microarray washing, staining and scanning

The washing and staining procedures were performed using the automated system “Fluidics Station 450/250” (Affymetrix, Santa Clara, USA). The Fluidics
Station is operated using the GCOS/Microarray Suite software (Affymetrix, Santa Clara, USA), which instruct the machine to perform standardised washing and staining steps according to the type of array used. Briefly, the fluidic station washes the hybridised arrays using the washing solutions, stains the hybridised aRNA by filling the array with the staining solution, expels the staining solution and fills the arrays with washing solution for scanning.

The composition of the solutions used for the washing and staining is reported below. Non-stringent wash buffer, for 1,000 mL: 300 mL 20X SSPE (3M NaCl, 0.2M NaH₂PO₄, 0.02M EDTA solution, BioWhittaker/Cambrex, Wokingham, UK); 1.0 mL 10% Tween-20 (Pierce, Rockford, USA); 699 mL of DEPC-treated water. The solution was filtered through a 0.2μm filter before use.

Stringent wash buffer, for 1,000 mL: 83.3 mL 12X MES stock buffer (see previous paragraph); 5.2 mL NaCl, 5M solution (Ambion, Austin, USA); 1.0 mL 10% Tween-20 (Pierce, Rockford, USA); 910.5 mL DEPC-treated water. The solution was filtered through a 0.2μm filter before use.

2X staining buffer, for 250 mL: 41.7 mL 12X MES stock buffer (see previous paragraph); 92.5 mL NaCl, 5M solution (Ambion, Austin, USA); 2.5 mL 10% Tween-20 (Pierce, Rockford, USA); 113.3 mL DEPC-treated water. The solution was filtered through a 0.2μm filter before use.

SAPE stain solution, for one array: 600 μL 2X staining buffer (see above); 48.0 μL BSA (50 mg/mL solution, Invitrogen Life Technologies, Carlsbad, USA); 12.0 μL Streptavidin Phycoerythrin (1 mg/mL SAPE solution, Molecular Probes, Invitrogen Life Technologies, Carlsbad, USA); 540.0 μL DEPC-treated water.

Antibody solution, for one array: 300.0 μL 2X staining buffer (see above); 24.0 μL BSA (50 mg/mL solution, Invitrogen Life Technologies, Carlsbad, USA); 6.0 μL goat IgG (10 mg/mL goat IgG stock solution: 50 mg goat IgG (Sigma-Aldrich) resuspended in 5 mL 150 mM NaCl); 3.6 μL anti-streptavidin goat biotinylated antibody (0.5 mg/mL solution, Vector Laboratories, Peterborough, UK); 266.4 μL DEPC-treated water.
After washing and staining, the arrays were scanned using GeneChip Scanner 3000 (Affymetrix, Santa Clara, USA). This machine is operated using the GCOS/Microarray Suite software (Affymetrix, Santa Clara, USA). The data are automatically acquired and stored for analysis.

2.2.11 Normalisation and analysis

After scanning, the image data were acquired and analysed using the GeneChip Operating Software (GCOS, Affymetrix). This software was also used to compute the signal intensities of each probe cell and the present calls.

The normalisation and statistical analysis of data were performed by Dr Pawel Herzyk at the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow.

The intensity values were normalised according to the Robust Multichip Average (RMA) method (Irizarry et al., 2003) implemented in module Affy in the Bioconductor microarray analysis software (http://www.bioconductor.org).

The normalised data were analysed to individuate the statistically significant expression differences according to the Rank Product (RP) method (Breitling et al., 2004). RP is a non-parametric, statistically rigorous method that is based on the calculation of rank products values, used to express the changed probe sets as a ranked list. This sophisticated methodology provides a straightforward way to select a reliable significance threshold on the basis of the False Discovery Rate (FDR), and therefore avoids the large numbers of false-positive results associated with traditional multiple t-tests. Additionally, it provides an estimate of the Fold Change (FC). The expression pattern of each region was compared to that of all the others.

The genes that showed localised expression were clustered in functional classes according to the "biological process" gene ontology. Annotations were
2.3 Confirmation of regional expression by *in situ* hybridisation

2.3.1 Probe design

\[^{38}S\] labelled oligonucleotide probes were used to validate the regional expression pattern of selected genes (table 4.1) predicted by the LMD/microarray study. Specific 45mer sequences were designed within the Affymetrix microarray target sequence corresponding to the gene of interest. This design strategy was chosen to ensure that the probes would detect the same mRNA as the microarray. The microarray target sequence was obtained from the NetAff database (www.affymetrix.com). The specificity of the 45mer probes was assessed using the Basic Local Alignment Search Tool (BLAST) software. The 45mers were compared by BLAST to the sequence databases available from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Ensembl project (www.ensembl.org). All the sequences showed specific alignment with the transcript of interest. Moreover, the secondary structure of the 45mer probes was predicted using the Oligo Calculator software (www.sigma-genosys.com). The sequences were accepted only when the predicted secondary structure was classified as "weak" or "moderate". The HPLC-purified 45mers were purchased from Thermo-Electron Corporation, Ulm, Germany.

2.3.2 Oligonucleotide labelling

The 45mer probes were labelled using a modification of the method described by Wisden and Morris (1994). 2.0 µl oligonucleotide (5 ng/µl) was incubated with
2µl terminal transferase reaction buffer (Roche Applied Science), 0.6µl cobalt chloride (25mM solution, Roche Applied Science), 2.5 µl [α-35S] dATP (specific activity 1250 Ci/mmol, NEN-PerkinElmer, UK), and 2.0 µl terminal transferase (TdT 25 units/µl, Roche Applied Science) at 37°C for 90 minutes. The reaction was stopped by addition of 60 µl DEPC-treated water. Labelled probes were purified using the QIAquick™ nucleotide removal kit (Qiagen). The activity of the probes was measured using a liquid scintillator counter (2200CA TriCarb, Packard). Probes with activity higher than 40,000 DPM/µl were hybridised on test sections (see below) to determine their specificity. Probes that produced a specific signal and low level of background were selected for hybridisation on experimental sections.

Probes were stored at -20°C after adding 4 µl of 1M DTT as stabiliser.

2.3.3 Slide preparation and fixation

The brains for the in situ hybridisation (ISH) experiments were obtained from six male Hooded Long Evans rats (Harlan, Oxon, UK) according to the protocol described in section 2.2.1. The animals’ age and weight matched with that of the ones used for the LMD-microarray experiment.

10 µm coronal sections were cut in a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany) at -20°C from the Bregma levels reported above and thaw-mounted on poly-L lysine coated glass slides (previously baked at 180°C and coated in a 0.61% poly-L-lysine solution made up in DEPC-treated water). The sections were air-dried at room temperature and fixed in a freshly prepared formaldehyde solution (4% w/v paraformaldehyde in phosphate buffered saline (PBS: 1.3M NaCl, 70mM Na2HPO4 and 30mM NAH2PO4 in DEPC-treated water) for 5 minutes, followed by washing in PBS for 5 minutes and sequential
dehydration in 70%, 95% and 100% ethanol (5 minutes each). The slides were then stored in absolute ethanol at 4°C until further processing.

2.3.4 Hybridisation and negative controls

Slides were removed from the absolute ethanol and air-dried at room temperature. The labelled probe was diluted in hybridisation buffer, and DTT was added as preservative. The hybridisation mixture for each slide was composed of: 4 µl probe, 16 µl DTT (1M solution in DEPC-treated water) and 200 µl of hybridisation buffer (50% deionised formamide, 4X SSC (3M NaCl, 0.3M sodium citrate treated with DEPC), 10% dextran sulphate, 0.5M sodium phosphate, 0.1M sodium pyrophosphate and 5 mg/ml polyadenylic acid; the final volume was adjusted with DEPC-treated water).

The negative control hybridisation mixture was prepared adding 16 µl cold probe (50 fold excess to the labelled probe).

The hybridisation mixture was applied to the slides and covered with Parafilm® to prevent dehydration. The slides were placed in a petri dish, with tissue soaked in 4X SSC to maintain humidity, and incubated at 42°C overnight.

Washing, development and image analysis

After overnight hybridisation, Parafilm® coverslips were removed in 1X SSC at room temperature, followed by a high stringency wash in 1X SSC at 60°C for 30 minutes. Slides were then washed at room temperature in 1X SSC, 0.1X SSC, 70% ethanol and 90% ethanol for 20 seconds each, followed by air-drying at room temperature. The slides were placed in X-ray cassettes and exposed to Biomax™MR films (Kodak) for 10-20 days.
Biomax™MR films were developed using an automatic developer (HyperProcessor, Amersham, Piscataway, NJ, USA).

Quantitative optical density measurements were taken using computer based densitometry (MCID-5, InterFocus Imaging Ltd, Linton, UK). The optical density was quantified in the regions corresponding to the LMD samples. Non-specific signal was subtracted from this value to obtain specific quantitative measurements. Non-specific signal was measured in brain regions that did not show expression, usually in the white matter. The measurements were defined as relative optical density.

2.3.5 Statistical analysis

The optical density values measured in the PrL, VO, M1 and Rt of the 6 biological replicates were compared using a within-subjects repeated measure ANOVA (repeated factor = brain region). In order to analyse the difference between regions, simple contrast was applied to the repeated measure ANOVA, setting one region as the control category. The control category region for each gene was the one identified as overexpressing the gene from the microarray (see section 3.3.5). This test allowed verification of whether the differences between the control category regions and all the others were statistically significant. The tests were performed using the SPSS statistics software package, version 11.0. Significance was accepted at P<0.05.
2.4 Expression of localised genes in a chronic PCP model of schizophrenia

2.4.1 Chronic PCP administration (chronic-intermittent PCP model of schizophrenia) and tissue preparation

Male hooded Long Evans rats (Harlan, Oxon, UK) were housed under a 12h light/dark cycle and received food and water ad libitum. The animals, weighing 180-220 g were treated with a daily i.p. injection of phencyclidine (PCP hydrochloride 2.58 mg/kg in 0.9 sterile saline n=6) or vehicle (0.9% sterile saline n=6) for 5 days. The rats were then injected with the same dose of PCP or saline on days 8, 10, 12, 15, 17, 19, 22, 24, 26.

72 hours after the last exposure to PCP (day 29), the animals were killed by cervical dislocation and decapitated. The brains were quickly removed and frozen in isopentane at -42°C. The brains were stored at -80°C until further processing. Experimental procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986 and associated guidelines.

2.4.2 In situ hybridisation

The sequences of the oligonucleotide probes used for this set of experiments (see chapters 5 and 6) are reported in tables 4.1 and 6.1. The probes specific for the genes described in chapter 5 were labelled according to the procedure described above. The same reaction was also used to label the probes specific for the genes described in chapter 6; however, these oligonucleotides were labelled
with $[\mu^{32}P]$ dATP (specific activity 3000 Ci/mmol, NEN-PerkinElmer, UK), since in this case this reagent allowed higher yields of radiolabelled probes. The experimental procedures for slide preparation, fixation, in situ hybridisation and image analysis are outlined in sections 2.3.3 and 2.3.4.

2.4.3 Statistical analysis

The relative optical density values measured in the PrL, VO, M1 and Rt (if detectable) of the PCP-treated animals were compared to those measured in the corresponding brain region of the vehicle-treated animals using an independent sample t-test. The test was performed using the SPSS statistics software package. Significance was accepted at $P<0.05$. 
CHAPTER 3. mRNA EXPRESSION PROFILING IN LASER-MICRODISSECTED BRAIN REGIONS
3.1 Introduction

3.1.1 Anatomical and functional heterogeneity of schizophrenia-related prefrontal cortical regions in the rat

The rat prefrontal cortex has generated wide interest in recent years, as the activation of this region is required for cognitive functions such as attentional processes, working memory and behavioural flexibility (Dalley et al., 2004). Despite the controversies about anatomical and functional homologies between brain structures of the prefrontal cortex in primates and rodents (Brown et al. 2002) (see also section 1.4.1.1), the use of rodent models has proved useful for the understanding of the pathophysiological processes that underlie psychiatric disorders such as schizophrenia, attention deficit/hyperactivity disorder (ADHD) and drug addiction (van den Buuse et al., 2005; Sagvolden et al., 2005) as well as the mechanisms of action of psychoactive drugs. Therefore basic research on the neurobiology of the rat prefrontal cortex has a multi-disciplinary value.

A number of recent studies focus on the functional heterogeneity of the prefrontal cortex, trying to associate specific aspects of cognitive and attentional processes to anatomical sub-regions (reviewed by Dalley et al. 2004). The rat prefrontal cortex can be anatomically subdivided into a dorsal medial region; a ventral medial region, which includes the prelimbic cortex (PrL) and the infralimbic cortex; a ventral region, including the ventral orbital cortex (VO); and a lateral region.

This study focuses on the PrL and the VO, as they are particularly relevant to aspects of modelling schizophrenia in rats. Recent findings suggest that the PrL is functionally analogous to the human dorsolateral prefrontal cortex (Floresco et al. 1997; Mizoguchi et al. 2000; Brown et al., 2002), which is one of the brain regions where schizophrenia-related neurochemical and molecular alterations are
more prominent (Harrison, 1999; Lewis et al., 1999). Moreover, a metabolic hypofunction has been reported in this area following chronic-intermittent phencyclidine (PCP) treatment in rats (Cochran et al., 2003). This region-specific decreased neuronal activity reproduces the metabolic hypofunction observed in the human prefrontal cortex, known as hypofrontality, which is a landmark of schizophrenia. Hypofrontality is considered to be associated with the presence and severity of negative symptoms and cognitive impairments (Hazlett et al., 2000; Buchsbaum et al., 1998; Cohen et al., 1997; Andreasen et al., 1992; Tamminga et al., 1992). The possibility to model this key neuropathological alteration in the rat PrL suggests that this region may be crucial for the cognitive abnormalities observed in the rat models of schizophrenia. Indeed, the chronic-intermittent PCP treatment regime (Cochran et al., 2003) also produces cognitive deficits in rats (Egerton et al., 2006).

The VO could also be crucial to models of schizophrenia as it controls impulsive choice behaviour (Mobini et al., 2002) and response selection (Chudasama et al. 2003a; Chudasama et al. 2003b). Furthermore, it has been shown that the VO has a role in the neuropathology of schizophrenia (Crespo-Facorro et al., 2001; Glahn et al., 2005). Specifically, studies based on PET (Positron Emission Tomography) have consistently shown decreased activity in the ventral prefrontal regions in the schizophrenic brain. The differences were observed both at rest and when performing cognitive tasks (Andreasen et al., 1997; Crespo-Facorro et al., 1999). Moreover, a study based on MRI (Magnetic Resonance Imaging) showed that the cortical surface of the orbital-frontal cortex is significantly reduced in drug-naïve schizophrenia patients in the early stage of the illness (Crespo-Facorro et al., 2000). This structural abnormality is very striking, as it is not caused by drug treatments or hypothetical neurodegenerative processes; therefore, the neuropathological alterations in the ventral region of the prefrontal cortex could also underlie aspect of the cognitive deficits observed in schizophrenia.

Interestingly, recent studies show that the PrL and the VO control different aspects of the attentional processes relevant to schizophrenia, suggesting a high
degree of functional specialisation for these subregions of the prefrontal cortex. The PrL is crucial for the extradimensional shift of the attentional set, which is the capability of shifting the attention from one perceptual dimension to another. In fact, lesions disrupting the PrL produce severe deficits in tasks testing the extradimensional set shifting. These deficits are not shown when the orbitofrontal cortex is disrupted (Dias et al., 1996; Dias et al., 1997). Conversely, lesions of the VO specifically impair reversal learning, which is the ability to re-learn the stimulus reward association under reversed reward values (Brown et al., 2002). These evidences are part of a growing body of information implying that the rat prefrontal cortex is a structure composed of interconnected functionally specialised subregions.

3.1.2 The thalamic reticular nucleus: modulation of the PFC-dependent cognitive processes

The reticular nucleus of the thalamus (Rt) is a thin sheet of neurons surrounding laterally the main body of the thalamus. The Rt is composed mainly of GABAergic interneurons, exerting an inhibitory control over the thalamo-cortical relay connections (Paxinos, 1995). The GABAergic interneurons of the Rt receive collateral inputs from both the cortico-thalamic and the thalamo-cortical fibers, and respond to these stimuli with an inhibitory modulation of the latter pathway (Paxinos, 1995).

The specific role of the Rt as a gateway for the flow of information between the thalamus and the cortex suggests that this nucleus could be crucial for those functions that require an accurate filtering of the stimuli, such as attentional processes. In fact, evidence from anatomical studies shows that the rostral part of the Rt directly controls the relay fibres connecting the mediodorsal thalamus and the prefrontal cortex (Cornwall et al., 1988). A more recent study shows that the
Rt activity is required when cognitively demanding spatial memory tasks are performed (Vann et al., 2000). These evidences, supported by observations from lesion studies (reviewed by McAlonan et al., 2002), strongly suggest that cognition and attention are controlled by a complex circuitry, of which the prefrontal cortex specific subregions are the core structures, and the regulation of their activity exerted by the Rt is crucial.

The strong evidence in favour of the importance of the Rt for the functions mediated by the prefrontal cortex, points towards a possible role of this thalamic nucleus in the neuropathological alterations observed when aspects of schizophrenia are modelled in rats. Strikingly, Cochran et al (2003) demonstrated that the metabolic hypofunction observed in the PrL of a rat model of schizophrenia, is also present in the Rt. The same study showed that the expression of parvalbumin, a calcium binding protein found in some specific subsets of GABAergic interneurons, is decreased in the Rt of the rat model of schizophrenia (see chapter 4).

3.1.3 From functional specificity to molecular characterisation: the importance of gene expression profiling in brain

One of the main goals of neuroscience research is the understanding of the structural and functional diversity of the brain both at the macroscopic and microscopic level. As shown by the literature, a massive effort has been put into associating functional specificity to anatomically distinct structures, employing a number of diverse approaches, such as lesion studies, functional neurochemistry, imaging techniques and behaviour studies.

The following step in the study of the modular organisation of the brain is identifying the association between functional specificity and molecular markers, such as gene expression profiles. Arguably, anatomical and functional diversity
should correspond to specificity at the molecular level, and the specific transcriptome of a region could be of paramount importance in this context. The gene expression profile of a brain region is likely to underlie a number of characteristics, such as cytoarchitecture, neuronal composition and neurochemistry; therefore the mRNAs expressed in a specific brain region constitute an informative molecular fingerprint that could be essential for its specific neurobiological functions.

3.1.4 Laser-assisted microdissection, linear amplification and microarray analysis: innovative tools for high-throughput expression analysis

The identification of molecular markers in rat brain regions can be complicated by several factors. The dissection of the functionally specialized cortical subregions for the downstream expression analysis requires a very high degree of anatomical precision. For this purpose conventional techniques, such as manual gross dissection, punch dissection and slices dissection, cannot guarantee that the specimens collected are free from unwanted material from neighbouring regions. Even a small amount of such contamination could introduce a significant bias that would affect the data interpretation altering their biological significance. The strict criteria for the dissection of cortical subregions and small thalamic nuclei can only be met by microscopy-based techniques that allow the exact identification of the structures of interest, coupled with a precise dissection system.

Laser-assisted microdissection (LMD) is a recently developed technique that can be used for the isolation of small structures, or even single cells, and can be successfully applied to neuroscience research (Bohm et al., 2005). Commercially available LMD instruments require a thin section of tissue (8-12 μm) mounted on a special slide and suitably stained for identification of the target structures; the
laser technologies employed for the dissection step can be based either on an infrared or an UV beam.

The system manufactured by Arcturus Engineering employs a cap of thermoplastic film, which is placed onto the tissue mounted on a glass slide. A low-energy infrared laser beam is focused through the microscope objective onto the film. When the laser is pulsed on a selected area of the section, it melts the film onto it. The selected tissue is then collected by lifting the cap. The thermoplastic film carries over only the specific area of tissue where the laser was pulsed. This technique is often referred to as Laser Capture Microdissection (LCM).

An alternative to the LCM system is the cold LMD technology, where a pulsed UV-A laser is used to cut around the selected target area. Instruments manufactured by Leica Microsystems and PALM Microlaser are based on this technology. The first microscope, which is the one employed in the present study, requires the section to be mounted on a membrane slide made of polyethylene terephthalate (PET). When the laser is pulsed around the selected area, it cuts through the tissue and the membrane; then the microdissected sample is collected by gravity into a tube cap placed under the microscope stage. This collection strategy does not require any additional manipulation or force application after pulsing the laser.

The LMD systems allow collection of samples in nuclease-free conditions; therefore, provided that all the procedures performed prior to the microdissection do not affect the RNA integrity, microdissected samples are suitable for expression analysis. The key step for the RNA integrity preservation is the staining. Microdissection of specific structures or cells requires histological or immunohistochemical staining for the identification of the target area. Ordinary staining protocols often involve the use of cross-linking fixatives, such as paraformaldehyde (PFA), and prolonged incubation in aqueous environments. These conditions are unsuitable if high-quality RNA needs to be extracted from the stained sample. Due to the increasing popularity of the LMD technique, some studies were specifically aimed at developing fixation and staining protocols that
would cause minimal damage to the RNA. Qin et al. (2003) compared expression profiles from PFA-fixed, paraffin embedded rat brain tissue, ethanol-fixed frozen rat brain tissue and non-fixed rat brain tissue. Van Deerlin et al (2002) performed a similar investigation using post mortem human brain tissue either flash-frozen of paraffin embedded with prior fixation in formalin or ethanol. Both studies show that the RNA integrity is well preserved when precipitative fixatives are used in frozen samples, whereas cross-linking fixatives and paraffin embedding produce less reliable results. Detailed studies comparing different staining methods have also been published (Ginsberg et al., 2004; Burbach et al., 2004), showing that the key factor for reliable RNA integrity preservation is reducing the staining time, especially when the samples must be incubated in aqueous environments. Most immunohistochemistry protocols developed for LMD are shorter than 30 minutes; acceptable staining can be achieved in these conditions by increasing the primary antibody concentration.

The amount of total RNA varies according to the type of tissue and the size of the microdissected area. However, it is not usually sufficient to perform high-throughput expression analysis, especially using microarray methodologies. For this reason, a massive effort has been put in developing an amplification strategy that would allow gene expression profiling of small samples. The key features of such a methodology are reliability and preservation of the relative abundance of the mRNA transcripts. In fact, a standard exponential amplification would alter the composition of the mRNAs, nullifying the biological significance of the downstream expression analysis. Suitable strategies for linear RNA amplification were first developed by Van Gelder et al. (1990) and Eberwine et al. (1992). Briefly, this approach is a linear, isothermal amplification based on in vitro transcription using T7 RNA polymerase where the mRNA is first reverse-transcribed into cDNA incorporating a functional T7-promoter, and then linearly amplified via in vitro transcription, obtaining amplified, antisense orientated RNA (aRNA). This procedure can be repeated to further increase the yield, and successfully maintain the original expression pattern (Poirier et al., 1997; Puskas et al., 2002). Many companies have developed commercially available kits for
linear amplification of RNA based on this strategy. Although the original method remains valid and reliable, Baugh et al. (2001) described a number of inconveniences that can occur especially when the amount of template is very low; such as the production of considerable amounts of non-template high molecular weight reaction artefacts and excessive shortening of the fragments. These problems can be circumvented with slight adaptations of the original method that have also been employed by the new generation commercial kits (see section 2.2.6 for details), making the procedure highly standardised and reliable.

The molecular characterisation of a specific tissue can be performed using a range of techniques to measure markers at the mRNA or protein level. Nevertheless, only the ability to obtain a simultaneous quantitative measurement of thousands of transcript can provide insightful information about the great complexity of specific brain regions. DNA microarray technology can be the answer to such technologically challenging investigation. DNA chips have been reliably used in recent studies for high-throughput expression analysis in different research fields, and their popularity is constantly increasing in neuroscience (Cao et al., 2001; Gebicke-Haerter, 2005; Konradi, 2005; Reimer et al., 2005). There are several different platforms that can be used for microarray-based expression analysis, ranging from self-spotted cDNA arrays to high-density oligonucleotide chips. Among them, the GeneChip technology developed by Affymetrix has been the platform of choice for many successful studies due to the vast amount of information produced and its reliability. The GeneChips consist of a solid support where DNA probes are chemically synthesized in situ. The highly standardised photolithographic process allows the synthesis of oligonucleotides (usually 25mers) at very high density on small individual arrays, which can measure simultaneously tens of thousands different transcripts in a sample. This technology also confers good reliability due to the standardisation of the productive process, as opposed to other types of platform that can suffer from some degree of variability (reviewed by Reimer et al., 2005).

The complex objective of individuating gene expression profiles in specific brain regions can be achieved thanks to the technological advantages of laser-
assisted microdissection, followed by linear amplification and microarray analysis. This powerful and innovative combination of techniques will be employed in the present study for an extensive analysis of the transcriptome of the rat PrL, VO and Rt.

3.1.5 Gene expression profiling via LMD/microarray in neuroscience research

Although the LMD/microarray strategy has been developed only recently, it soon captured the interest of neuroscientists due to its impressive potential. A number of studies have been published recently (reviewed by Bohm et al., 2005), some of which focus on the optimisation and technical aspects of the process (Sanna et al., 2005). The attempts to identify gene expression profiles are in many cases focused on the study of the hippocampal region of both humans and rats (Bonaventure et al., 2002; Datson et al., 2004; Torres-Munoz et al., 2004). The hippocampus is particularly suited for this type of analysis as the subregions are easily identifiable after quick staining and are mainly composed of discrete and uniform layers of large pyramidal neurons; moreover, the functions of hippocampal subregions are well studied. Datson et al. (2004) demonstrated the feasibility of gene expression profiling in rat hippocampal subregions using the Leica AS LMD microdissection system and Affymetrix GeneChip arrays. This study focused on the expression comparison between the cornu ammonis 3 (CA3) and the dentate gyrus (DG) subregions, finding a substantial differential expression underlying morphological and functional differences. Specific expression pattern in closely related hippocampal subregions were also reported by Bonaventure et al. (2002) using the Arcturus LCM system. The amygdala is another region where molecular characterization has been performed using LMD/microarray approaches (Zirlinger et al., 2000; Zirlinger, 2003). This study
also employs the Arcturus LCM system to dissect specific amygdaloid subnuclei, where differential gene expression was demonstrated.

Studies of the hippocampus and amygdala show the vast potential of the LMD/microarray approach for the construction of expression profile databases of specific brain regions. However, this novel technology can be implemented especially by the development of new, more comprehensive and robust microarray platforms. Further research is also needed to improve the reproducibility and robustness of the findings, as well as expanding the current knowledge to other interesting brain areas.

Although Sanna et al. (2005) demonstrated the technical feasibility of expression profiling of rat thalamic nuclei, the literature is currently lacking detailed information about genes specifically expressed or enriched in these regions. Also, the LMD/microarray approach has not been applied for the study of specific cortical subregions. The molecular characterization of the cortex is a challenging task, due to the cytoarchitectural and functional complexity of this area. In contrast to the hippocampus and other well-defined brain regions, cortical subregions are not characterized by the prevalence of one neuronal population, but consist of a complex structure composed of diverse neuronal subtypes. The global transcription profile of such structures represents the phenotype of their complex neurocircuitry and could underlie the neurobiology of higher cognitive functions.
3.2 Aims

This study has multiple aims. Firstly, it will test the technical feasibility of molecular characterization of rat cortical subregions and thalamic nuclei using an approach based on laser-assisted microdissection, linear amplification and microarray analysis. The gene expression profiling will be performed on the Prelimbic cortex (PrL), Ventral Orbital cortex (VO) and Reticular Thalamic nucleus (Rt) for their relevance to cognitive processes and models of schizophrenia, and additionally on the Primary Motor cortex (M1) as a control region.

Another aim is to test the hypothesis that the brain regions analysed show significant differential gene expression, reflecting structural and functional differences. The expression profiles of the four regions will be compared to assess both the variation between thalamus and cortex and the intra-cortical differential expression.

Finally, the genes that showed localised expression will be clustered into functional classes according to the “biological process” gene ontology. This analysis will be based on information available from the literature and online databases, and will facilitate the identification of localised groups of functionally related genes. The expression profile of the M1 will be included in this analysis for comparative purpose. The M1 is a neocortical region not directly involved in attention, cognition and memory, therefore the comparison of the expression profile of this region to that of the PrL and the VO will help in the identification of gene clusters hypothetically involved in those functions.
3.3 Results

3.3.1 Tissue staining and microdissection

The quick staining protocol optimised for the LMD allowed the unambiguous identification of the cortical subregions on the sections mounted on PET slides (figure 3.1). Even after dehydration, the toluidine-blue staining allowed the visualization of specific structures and landmarks of the neocortex, such as the white matter and the cortical layers. At higher magnification, single neurons were also easily recognisable.

The immunohistochemical staining of the anterior thalamus was very helpful for the identification of the Rt in the adjacent toluidine-blue stained sections. Although GAD65/67 immunoreactivity is present in other thalamic nuclei, the prevalence of GABAergic neurons in the Rt produced a remarkably stronger staining compared to the neighbouring regions (figure 3.2A). The structure showing strong GAD65/67 immunoreactivity could be clearly identified in the toluidine-blue stained section (figure 3.2B).

The LMD procedure was efficient and reliable for the microdissection of the required brain regions with a high degree of anatomical precision (figures 3.1 and 3.2). All dissections were performed efficiently according to a conservative interpretation of the anatomical boundaries.
Figure 3.1
Toluidine blue-stained rat brain section at the prefrontal cortex level (Bregma 3.70 mm). The magnified photographs show part of the section after laser-assisted microdissection of M1 (1), PrL (2) and VO (3). (Al: agranular insular cortex; Cg1: cingulate cortex area 1; Cl: claustrum; LO: lateral orbital cortex; M1, primary motor cortex; M2, secondary motor cortex; MO: medial orbital cortex; OB: olfactory bulb; PrL: prelimbic cortex; VO: ventral orbital cortex; WM: white matter).
Figure 3.2

A. GAD 65/67 immunoreactivity in the reticular thalamic nucleus (Rt) (Bregma -1.60 mm). The GABAergic interneurons of the Rt show stronger immunoreactivity than the neighbouring regions.

B. Toluidine blue-stained rat brain section, anterior thalamus level (Bregma -1.60 mm). Comparison with the adjacent immunostained section (A) facilitates the identification of the Rt.

C. Toluidine blue-stained rat brain section, anterior thalamus level, with the Rt highlighted (Bregma -1.60 mm). The magnified photograph shows part of the section after laser-assisted microdissection of the Rt. (Amy: amygdala; ant/vent thalamus: anterior/ventral thalamic nuclei; CA3, field CA3 of hippocampus; ec: corpus callosum; cg: cingulum; Cpu: caudate putamen; D3V: dorsal 3rd ventricle; eo: external capsule; fi: fimbria of hippocampus; HYP: hypothalamus; ic: internal capsule; LGP: lateral globus pallidus; sm: stria medullaris of thalamus).
3.3.2 Total RNA quality control

The electropherograms showed that the degree of degradation of the total RNA extracted from the LMD samples was negligible (figure 3.3). In all samples the peaks corresponding to the 18S and 28S rRNA were clearly visible and had similar height. The intensity of the signal corresponding to fragmented RNA was very low. These results showed that the quality of the RNA obtained was very high; therefore all samples were suitable for the downstream processing and gene expression analysis.

The RNA 6000 Pico LabChip kit does not provide an accurate quantitative measure of the total RNA; however the estimated values suggested that each sample contained from 10 to 20 ng of total RNA.

3.3.3 Amplified aRNA quality control

The two-round linear amplification procedure yielded on average 45 µg of biotin-labelled aRNA. As expected, the electropherograms of all samples showed a fragment size range from 200 to 3000 nucleotides (nt), with a maximum peak at 800 nt (figure 3.4). The negative control amplification products showed very low concentration of small fragments, (fragments size <200 nt) (figure 3.4); probably reflecting the presence of carriers used during the purification steps. The amount of this material was below the sensitivity limit of the spectrophotometer. The absence of a significant amount of material in the negative controls and the uniformity of the samples suggested that the linear amplification procedure was specific and reproducible and that the labelled aRNA was free of reaction artefacts (Baugh et al. 2001).
Figure 3.3
Representative electropherograms of total RNA extracted from LMD samples (brain regions are specified in the top right corner). The peaks corresponding to the 18S and 28S rRNA show that the quality of the RNA obtained was very high.
Figure 3.4
Representative electropherogram of biotin-labelled aRNA (1:5 dilution) (brain regions are indicated in the top right corner), and electropherogram of no template negative control (ncat). The qualitative and quantitative difference between the sample and the negative control shows that the labelled aRNA was free of reaction artefacts.
3.3.4 Microarray hybridisation

The average percentage of probes called present by the GCOS software was 51.6% in the M1, 52.3% in the PrL, 49.0% in the VO and 43.5% in the Rt. The slightly higher percentage of transcript detected in the cortical areas could mirror the higher degree of complexity and cellular heterogeneity of the cortex in comparison to a relatively simple structure as the Rt.

To assess the reproducibility of the LMD-linear amplification-microarray analysis strategy, scatter plots of signal intensities were constructed and correlation coefficients were calculated between all samples. The Pearson correlation coefficients between biological replicates ranged from 0.972 to 0.995 (figure 3.5). The correlation coefficients between samples from different cortical regions were also very high, ranging from 0.961 to 0.990. When the signal intensities obtained from cortical regions were correlated with those from the Rt, the coefficients ranged from 0.903 to 0.955 (figure 3.6).

The high percentage of transcripts detected in all the specific brain regions and the strong correlation between replicate samples supports the validity and the reliability of the LMD-linear amplification-microarray experimental strategy. Also, the Pearson correlation analyses show that the gene expression differences between the Rt and the cortical regions are greater than the intra-cortical variations.
Figure 3.5
Scatterplots of normalised signal intensity (RMA method) of probe sets from representative biological replicates (regions are indicated by the axis legend). The Pearson correlation coefficients between biological replicates ranged from 0.972 to 0.995.
Figure 3.6
Figure 3.6
Scatterplots of normalised signal intensity (RMA method) of probe sets from representative samples (regions are indicated by the axis legend). The correlation coefficients between samples from different cortical regions ranged from 0.961 to 0.990, whereas those calculated comparing the Rt to cortical regions ranged from 0.903 to 0.955. The correlation analyses show that the gene expression differences between the Rt and the cortical regions are greater than the intra-cortical variations.
3.3.5 Regional differential gene expression

The ranked lists of differentially expressed probes generated by the RP analysis were combined to identify the transcripts enriched in one region compared to the others. This approach highlights the genes that are significantly overexpressed and, in some cases, specifically expressed in one region. Those genes could be relevant to the neurobiology of the region where they are enriched, as they may be essential for the specific functions of the region. This study focused on the specific gene expression pattern of the PrL, the VO and the Rt, since these regions show important changes in rat model of schizophrenia (Cochran et al. 2003) and play a key role in several aspects of cognition and attention that are relevant to modelling psychiatric disorders. Conversely, the M1 is not directly involved in attentional and cognitive processes and does not show prominent changes in the schizophrenic brain or in animal models; therefore its expression pattern was used for comparative analysis.

It was decided to consider all the probe sets that showed differential expression with a false discovery rate (FDR, see section 2.2.11) ≤ 1%; therefore transcripts that were enriched in one region with an FDR below this threshold are defined as overexpressed. This threshold was conservative enough to keep to a minimum the number of false positives, while including a considerable number of probe sets to avoid the loss of important information.

When the Rt was compared to the PrL, VO and M1, 429 transcripts were overexpressed (table 3.1), whereas 881 were enriched in all three cortical areas. The gene expression difference is substantial, especially considering that 206 of the Rt-enriched genes (48%) showed a Fold Change greater than 3. These results suggest that the examined cortical areas express a wider range of genes than the Rt. This could reflect a higher diversity in terms of neuronal population and more complex functions for the cortical areas. On the other hand, the genes overexpressed in the Rt could be crucial for the specific functions of this area, such as the inhibitory control over the thalamo-cortical relay projections.
Intra-cortical expression differences were also substantial. 233 genes were enriched in the PrL compared to VO and M1; whereas 83 genes were enriched in the VO compared to PrL and M1 (table 3.1). The intra-cortical differential expression is less prominent than that observed when the Rt is compared to the cortical regions. This could be due to the fact that the cortical regions have a similar cytoarchitecture and neuronal composition. Interestingly, the PrL showed a more pronounced overexpression of genes than the VO. This finding could suggest that the PrL has higher functional complexity than the other cortical areas examined in this study.

<table>
<thead>
<tr>
<th>Overexpressing region</th>
<th>Comparison regions</th>
<th>M1</th>
<th>VO</th>
<th>M1+VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrL</td>
<td></td>
<td>431</td>
<td>442</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PrL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1+PrL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO</td>
<td></td>
<td>213</td>
<td>460</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PrL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1+PrL+VO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rt</td>
<td></td>
<td>730</td>
<td>637</td>
<td>650</td>
</tr>
</tbody>
</table>

Table 3.1
Gene expression profile comparisons. Number of transcripts overexpressed (False Discovery Rate < 1%) in the Prelimbic cortex (PrL, upper row), Ventral Orbital cortex (VO, middle row) and Reticular Thalamic nucleus (Rt, bottom row) compared to the regions indicated in each column. (M1= Primary Motor cortex).
3.3.6 Biological function of localised genes

The region-enriched genes clustered into several functional classes. Different genes directly involved in synaptic transmission were selectively expressed in all the examined regions. Some GABAergic machinery-related genes, such as the two isoforms of glutamate decarboxylase (GAD65 and GAD67), were overexpressed in the Rt (see table 3.2, “Neurotransmission” functional class), in accordance with the prevalence of GABAergic interneurons in this region. Another interesting finding was the different distribution of GABA_A receptor subunits in the cortical regions. The α5 and the β1 subunits were overexpressed in the PrL (see table 3.3, “Neurotransmission” functional class), whereas the δ subunit was predominant in the VO (see table 3.4, “Neurotransmission” functional class).

Genes controlling ion transport were also selectively expressed in all regions. Specifically, the Rt and the VO showed overexpression of a number of potassium channel subunits (tables 3.2 and 3.4); whereas the PrL overexpressed genes involved in the transport of calcium and sodium (table 3.3).

Differential expression of genes related to cell adhesion, metabolism, cell cycle, phosphorylation and transcription was also prominent, especially in the Rt (table 3.2). The calcium-binding proteins parvalbumin and calretinin, typically expressed in the GABAergic interneurons, were also predominant in the Rt (see table 3.2, “Calcium binding proteins” functional class).

Another interesting finding was the overexpression of nine genes directly involved in neurogenesis and neurodevelopment in the PrL (see table 3.3, “Neurogenesis/neurodevelopment” functional class). This is a unique feature of this region, as neither the Rt nor the VO showed overexpression of genes involved in the regeneration or remodelling of the neural circuitry.
<table>
<thead>
<tr>
<th>Functional class</th>
<th>Probe set ID</th>
<th>Gene Title</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion</td>
<td>1374248_at</td>
<td>myosin binding protein C, slow type</td>
<td>16.30</td>
</tr>
<tr>
<td></td>
<td>1370889_at</td>
<td>ret proto-oncogene</td>
<td>13.03</td>
</tr>
<tr>
<td></td>
<td>1370697_a_at</td>
<td>nexilin</td>
<td>4.89</td>
</tr>
<tr>
<td></td>
<td>1369425_at</td>
<td>cadherin 13</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td>1369861_a_at</td>
<td>myelin-associated glycoprotein</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>1371112_at</td>
<td>ret proto-oncogene</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>1368135_at</td>
<td>ninjurin 2</td>
<td>2.62</td>
</tr>
<tr>
<td>Calcium binding proteins</td>
<td>1370214_at</td>
<td>parvalbumin</td>
<td>9.50</td>
</tr>
<tr>
<td></td>
<td>1370849_at</td>
<td>Hyaluronan and proteoglycan link protein 2</td>
<td>3.61</td>
</tr>
<tr>
<td></td>
<td>1368133_at</td>
<td>calbindin 2 (calretinin)</td>
<td>2.79</td>
</tr>
<tr>
<td></td>
<td>1368044_at</td>
<td>secretogranin 2</td>
<td>3.16</td>
</tr>
<tr>
<td>Inositol phosphate mediated signalling</td>
<td>1369345_at</td>
<td>inositol polyphosphate-4-phosphatase, type II, 105kD</td>
<td>10.08</td>
</tr>
<tr>
<td></td>
<td>1368119_at</td>
<td>phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A</td>
<td>2.92</td>
</tr>
<tr>
<td>Neurotransmission</td>
<td>1369462_at</td>
<td>glutamate decarboxylase 2 (GAD67)</td>
<td>6.63</td>
</tr>
<tr>
<td></td>
<td>1368475_at</td>
<td>collagen-like tail subunit (single strand of homotrimer) of asymmetric acetylcholinesterase</td>
<td>6.17</td>
</tr>
<tr>
<td></td>
<td>1367114_at</td>
<td>protein kinase C, delta</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>1369926_at</td>
<td>glutathione peroxidase 3</td>
<td>7.83</td>
</tr>
<tr>
<td></td>
<td>1369799_at</td>
<td>4-aminobutyrate aminotransferase</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>1369384_at</td>
<td>glutamate receptor, ionotropic, 4 (GluR4)</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>1368344_at</td>
<td>glutamate decarboxylase 1 (GAD65)</td>
<td>2.50</td>
</tr>
<tr>
<td>Ion transport</td>
<td>1368574_at</td>
<td>potassium voltage gated channel, Shaw-related subfamily, no. 1</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>1367815_at</td>
<td>solute carrier family 5 (sodium-dependent vitamin transporter) n. 6</td>
<td>2.48</td>
</tr>
<tr>
<td></td>
<td>1369687_at</td>
<td>potassium voltage gated channel, shaker related subfamily, beta n. 3</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>1367853_at</td>
<td>solute carrier family 12, n. 2</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>1368343_at</td>
<td>potassium voltage-gated channel, subfamily H (eag-related), n. 2</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>1370076_at</td>
<td>potassium inwardly-rectifying channel, subfamily J, n. 16</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>1360945_at</td>
<td>Transient receptor potential cation channel, subfamily C, n. 7</td>
<td>2.10</td>
</tr>
<tr>
<td>Functional class</td>
<td>Probe set ID</td>
<td>Gene Title</td>
<td>Fold Change</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Structural constituent of myelin sheath</td>
<td>1370506_a_at</td>
<td>myelin-associated oligodendrocytic basic protein</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>1398257_at</td>
<td>myelin oligodendrocyte glycoprotein</td>
<td>2.30</td>
</tr>
<tr>
<td>G protein mediated signalling</td>
<td>1387182_at</td>
<td>G protein-coupled receptor 37 (endothelin receptor type B-like)</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>1368705_at</td>
<td>endothelial differentiation, sphingolipid G-protein-coupled receptor, 8</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>1367957_at</td>
<td>regulator of G-protein signalling 3</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>1382967_at</td>
<td>G protein-coupled receptor 64</td>
<td>1.97</td>
</tr>
<tr>
<td>Transcription/ RNA processing</td>
<td>1369681_at</td>
<td>ISL1 transcription factor, LIM/homeodomain 1</td>
<td>5.01</td>
</tr>
<tr>
<td></td>
<td>1370083_at</td>
<td>nuclear receptor subfamily 2, group F, member 2</td>
<td>4.55</td>
</tr>
<tr>
<td></td>
<td>1368933_at</td>
<td>adenosine deaminase, RNA-specific, B1</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>1370089_at</td>
<td>peroxisome proliferative activated receptor, gamma, coactivator 1</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>1369690_a_at</td>
<td>DNA-damage inducible transcript 3</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>1388133_at</td>
<td>PIP5In protein</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>1371890_at</td>
<td>Similar to PKR-associated protein RAX</td>
<td>2.00</td>
</tr>
<tr>
<td>Metabolism</td>
<td>1368075_at</td>
<td>lipase A, lysosomal acid</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>1370387_at</td>
<td>cytochrome P450, family 3, subfamily a, polypeptide 13</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>1386554_at</td>
<td>adenylate kinase 2</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>1367735_at</td>
<td>acetyl-Coenzyme A dehydrogenase, long-chain</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>1386660_at</td>
<td>Pyrophosphatase</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>1388960_at</td>
<td>fumarylacetoacetate hydrolase</td>
<td>1.87</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>1367813_at</td>
<td>protein phosphatase 1, regulatory (inhibitor) subunit 14A</td>
<td>3.19</td>
</tr>
<tr>
<td></td>
<td>1367114_at</td>
<td>protein kinase C, delta</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>1368913_at</td>
<td>phosphatidic acid phosphatase type 2c</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>1374310_at</td>
<td>protein phosphatase 2a, catalytic subunit, zeta isoform</td>
<td>2.57</td>
</tr>
<tr>
<td>Functional class</td>
<td>Probe set ID</td>
<td>Gene Title</td>
<td>Fold Change</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------------</td>
<td>------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Cytoskeleton organisation and biogenesis</td>
<td>1370933_at</td>
<td>myosin IE</td>
<td>5.12</td>
</tr>
<tr>
<td></td>
<td>1370815_at</td>
<td>neurofilament, heavy polypeptide</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>1387157_at</td>
<td>polyamine modulated factor 1 binding protein 1</td>
<td>2.27</td>
</tr>
<tr>
<td>Cell cycle/apoptosis</td>
<td>1371643_at</td>
<td>cyclin D1</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>1371112_at</td>
<td>ret proto-oncogene</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>1367859_at</td>
<td>transforming growth factor, beta 3</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>1367957-a_at</td>
<td>SH3-domain kinase binding protein 1</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Table 3.2
Genes clustered according to similar biological function that are enriched in the Reticular Thalamic nucleus (Rt). All the transcripts showed FDR< 1%. The Fold-change values refer to the mRNA level difference between the Rt and the M1.
<table>
<thead>
<tr>
<th>Functional class</th>
<th>Probe set ID</th>
<th>Gene Title</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurogenesis/Neurodevelopment</td>
<td>1387141_at</td>
<td>Dihydropyrimidinase-like 5 (DPYSL5)</td>
<td>10.21</td>
</tr>
<tr>
<td></td>
<td>1369999_a_at</td>
<td>Neuronatin</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td>1367888_at</td>
<td>MT-protocadherin</td>
<td>2.92</td>
</tr>
<tr>
<td></td>
<td>1388101_at</td>
<td>Dihydropyrimidinase-like 3 (DPYSL3)</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>1369358_e_at</td>
<td>Huntingtin-associated protein 1</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>1368677_at</td>
<td>Brain derived neurotrophic factor (BDNF)</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>1368358_a_at</td>
<td>Protein tyrosine phosphatase, receptor type, R</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>1379374_at</td>
<td>Plasticity related gene 1</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>1374966_at</td>
<td>Doublecortin</td>
<td>1.50</td>
</tr>
<tr>
<td>Ion transport</td>
<td>1370248_at</td>
<td>FXYD domain-containing ion transport regulator 6</td>
<td>10.21</td>
</tr>
<tr>
<td></td>
<td>1368339_at</td>
<td>Wolfmin</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>1369691_at</td>
<td>Sodium channel, voltage-gated, type III, alpha polypeptide</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>1369160_a_at</td>
<td>Solute carrier family 4, sodium bicarbonate cotransporter, no. 7</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>1368368_at</td>
<td>Calcium channel, voltage-dependent, T type, alpha 1H sub</td>
<td>1.64</td>
</tr>
<tr>
<td>Neurotransmission</td>
<td>1368478_at</td>
<td>Dopamine receptor IA</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>1369019_at</td>
<td>Cholinergic receptor, nicotinic, alpha polypeptide 5</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>1370431_at</td>
<td>Synapsin II</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>1387698_a_at</td>
<td>Glycine receptor, alpha 2 subunit</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>1376345_at</td>
<td>Dopamine receptor D1 interacting protein</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>1371057_at</td>
<td>Gamma-aminobutyric acid A receptor, alpha 5</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>1339004_at</td>
<td>Gamma-aminobutyric acid receptor, subunit beta 1</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>1367977_at</td>
<td>Synuclein, alpha</td>
<td>1.63</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>1370312_at</td>
<td>F-spondin</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>1379350_at</td>
<td>Limbic system-associated membrane protein</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>1368936_at</td>
<td>Cadherin 11</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>1384734_at</td>
<td>Neural cell adhesion molecule 2</td>
<td>1.65</td>
</tr>
</tbody>
</table>

Table 3.3

Genes clustered according to similar biological function that are enriched in the Prelimbic cortex (PrL). All the transcripts showed FDR < 1%. The Fold-change values refer to the mRNA level difference between the PrL and the M1.
<table>
<thead>
<tr>
<th>Functional class</th>
<th>Probe set ID</th>
<th>Gene Title</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotransmission</td>
<td>1368564_at</td>
<td>solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>1369048_at</td>
<td>gamma-aminobutyric acid A receptor, delta</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>1368585_at</td>
<td>cocaine and amphetamine regulated transcript</td>
<td>1.51</td>
</tr>
<tr>
<td>Transcription/ RNA</td>
<td>1388133_at</td>
<td>PIPPin protein</td>
<td>1.90</td>
</tr>
<tr>
<td>processing</td>
<td>1387260_at</td>
<td>Kruppel-like factor 4 (gut)</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>1369007_at</td>
<td>nuclear receptor subfamily 4, group A, member 2</td>
<td>1.54</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>1378209_a_at</td>
<td>serine/threonine kinase 23</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>1370852_at</td>
<td>neurotrophic tyrosine kinase, receptor, type 2</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>1370853_at</td>
<td>CaM-kinase II inhibitor alpha</td>
<td>1.51</td>
</tr>
</tbody>
</table>

Table 3.4
Genes clustered according to similar biological function that enriched in the Ventral Orbital cortex (VO). All the transcripts showed FDR < 1%. The Fold-change values refer to the mRNA level difference between the VO and the M1.
3.4 Discussion

The combination of laser assisted microdissection, linear amplification and microarray has been shown to be a reliable method to identify gene expression differences in discrete regions of the rat brain, such as the hippocampus and the amygdala (Bonaventure et al. 2002; Datson et al. 2004; Sanna et al. 2005). In this study the LMD-microarray approach was used for the first time to perform multiple comparison of the gene expression profiles from two subregions of the prefrontal cortex and one thalamic nucleus involved in the neurobiological changes observed in a rodent PCP model of schizophrenia (Cochran et al. 2003).

The high percentage of mRNAs detected by the microarray and the uniformity of the data confirm the consistency of the technique and shows that it can be reliably applied to analyse the gene expression in cortical subregions. The differential expression was substantial when the Rt was compared to the cortical regions, as expected from the very different cytoarchitecture and neuronal composition. The differential expression was also prominent and reliably detected when the cortical subregions were compared to each other. This finding showed that the gene expression profile of a brain region does not depend exclusively on the gross cytoarchitecture and neuronal composition, which are relatively uniform in the cortical subregions studied. The mRNAs expressed in a specific cortical region constitute a molecular fingerprint that could reflect the presence of specific neuronal subtypes, the organisation of the synaptic connections, and could be essential for its specific neurobiological functions.

This study shows that the vast potential of the LMD-microarray approach can be applied to analyse expression differences between subregions of the neocortex that have similar cytoarchitecture and neuronal composition, but different functions. Understanding the role of the genes enriched in the PrL and the VO will clarify the neurobiological basis of functions such as attentional processes, executive function, working memory and behavioural flexibility, which are
affected in psychiatric diseases such as schizophrenia and drug addiction, and in animal models reproducing aspects of these conditions (Lee et al., 2004; Chudasama and Robbins, 2004; Egerton et al., 2005; Morey et al., 2005; Conklin et al., 2005; Kalivas et al., 2005). The genes enriched in the Rt are also crucial for the modulation of cortical activity, as they are likely to be the molecular basis of the inhibitory control over the thalamo-cortical relay pathways exerted by the Rt.

3.4.1 Functional classes of localised genes

The clustering of localised genes into functional classes highlighted that some specific biological pathways could be predominantly active in some regions.

The overexpression of genes with common function in a specific region can be due to the prevalence of a neuronal subpopulation or to the presence of anatomical structures such as white matter fibres. This interpretation is consistent with the biological characteristics of the Rt (see below).

When the neuronal composition and the anatomy of the regions are more uniform, like in the case of cortical subregions, these factors are likely to underlie the differential gene expression to a lesser extent. In this scenario, the differential expression of functional classes of genes could reflect how similar neuronal subtypes interact, forming complex networks in order to control specific functions. The organisation of the neurocircuitry and the inputs that neurons receive both from neighbouring cells and other brain structures are likely to have a relevant influence over the functional differences between specific brain regions, which can be mirrored at the molecular level by the differential gene expression observed in this study.
3.4.2 Overexpression of neurodevelopment-related genes in the PrL

An interesting finding is the overexpression of nine genes directly involved in neurogenesis or neurodevelopment in the PrL (table 3.3).

Dihydropyrimidinase-like 3 (DPYSL3) and dihydropyrimidinase-like 5 (DPYSL5), two genes belonging to the family of collapsin response mediator proteins, are enriched in this area; with DPYSL5 showing a FC of 10.21. Such a fold change value measured between cortical subregions with similar anatomy is a striking finding, as expression variations in the brain are usually small; therefore DPYSL5 could be considered a PrL-specific gene. The collapsin response mediator proteins have been implicated in developmental processes such as signalling of axonal guidance (Nacher et al. 2000; Hotta et al. 2005); DPYSL3 is expressed in the areas that show adult neurogenesis, such as the subventricular zone and the granular layer of the dentate gyrus (Nacher et al. 2000). Doublecortin, another gene directly involved in different aspects of neurodevelopment (Gupta et al. 2002; Friocourt et al. 2003) was also overexpressed in the PrL. The role of this gene in the development of mammalian cortex is well documented, as mutations are known to cause lissencephaly in humans, which is a type of cortical dysplasia (des Portes et al., 1998; Gleeson et al., 1998). The main function of doublecortin is to direct neuronal migration by interacting with the microtubules during the polymerisation process (Gleeson et al., 1999; Francis et al., 1999; Horesh et al., 1999). Although the overexpression of doublecortin in the PrL is less prominent than that of DPYSL5, (FC 1.50 as compared to 10.21), the significant prevalence of a gene with such a specific role in neurodevelopment in the PrL is likely to be biologically relevant.

The prevalence of neurodevelopment-related genes in the PrL is supported by the overexpression of brain derived neurotrophic factor (BDNF) (table 3.3). The role of this neurotrophin in neurogenesis and development is well documented (reviewed by Thomas et al., 2005), as well as its involvement in a number of
psychiatric diseases, such as schizophrenia, depression and eating disorders (Durany et al., 2004; Newton et al., 2004; Hashimoto et al., 2005).

It is difficult to predict how these genes interact and their role in a region that does not show incontrovertible evidence of neurogenesis. It could be argued that the overexpression of development-related genes in the PrL is linked to the fact that this is the last neocortical area to complete development; in fact in humans the PFC does not become mature until adolescence. Nevertheless, this explanation is not very probable, as the animals used in this study were fully developed adult rats (see materials and methods, chapter 2). On the other hand, the overexpression of this class of genes in the PrL suggests that the neural network of this area is extremely dynamic and active. Interestingly, some of these genes are also expressed in the hippocampus, and could be crucial for the higher cognitive functions of these structures.

It has been proposed that neurotrophins play a key role in memory and learning, as these molecules are involved in synaptic plasticity (reviewed by Lu, 2003). Interestingly, there is evidence of a reciprocal regulation between neurotrophin expression and synaptic activity (Thoenen, 1995; Berninger et al., 1996; Lu et al., 1997). Many studies investigating the role of neurotrophins in cognition focus on BDNF, which is overexpressed in the PrL according to the present work. This molecule has an acute effect on neurotransmission in vitro; in particular, it enhances neurotransmitter release at neuromuscular synapses (Lohof et al., 1993). Other groups investigated the acute effect of BDNF on hippocampal synapses, and found that this neurotrophin facilitates the release of neurotransmitter via a presynaptic mechanism and regulates long-term potentiation (Figurov et al., 1996; Patterson et al., 1996). Interestingly, the acute modulation of presynaptic activity exerted by BDNF is due to its control over the phosphorylation state of proteins involved in the docking of synaptic vesicles, as demonstrated in isolated synaptosomes from rodent cortex (Jovanovic et al., 2000). This work showed that the BDNF dependent MAP kinase activation leads to the phosphorylation of synapsins, a group of presynaptic proteins that control the proportion of vesicles available for exocytosis in the presynaptic terminal (see
chapter 4). The activity of synapsins is finely modulated by their phosphorylation state; therefore BDNF acutely enhances the release of neurotransmitters by controlling the phosphorylation of synapsins (Jovanovic et al., 2000). This finding is supported by the lack of BDNF dependent neurotransmission enhancement in mice lacking the synapsin I and/or synapsin II genes (Jovanovic et al., 2000). It is interesting that the LMD/microarray study also detected an overexpression of synapsin II in the PrL (see also chapter 4); therefore two genes that participate in the presynaptic control of long-term potentiation are specifically enriched in this cortical subregion, and are likely to be directly involved in the molecular mechanisms underlying PrL-dependent cognitive processes.

The neurotrophin-mediated control of synaptic transmission is not limited to an acute effect over the synaptic vesicles release; in fact BDNF has been shown to exert a complex long-term regulation over synapse development. This molecule has been implicated in processes such as dendritic and axonal growth in different brain areas, including new synapse formation in glutamatergic and GABAergic neurons of the hippocampus (Cohen-Cory et al., 1995; Rutherford et al., 1998; Vicario-Abejon et al., 1998; Sherwood et al., 1999).

More evidence for the involvement of neurotrophins in cognition derives from research investigating the activity-dependent synaptic expression of these molecules. Again, many studies focus on BDNF, finding that the expression of the mRNA encoding for this factor is increased in the hippocampus and prefrontal cortex following exposure to enriched environment (Young et al., 1999; Ickes et al., 2000; Zhao et al., 2001), sensory stimulation (Rocamora et al., 1999; Nanda et al., 2000) and also physical activity and running (Berchtold et al., 2001).

Although the mechanism underlying the regulation of BDNF transcription under these conditions and their functional significance are not fully understood, these fascinating observations suggest there is a reciprocal control between the neurotrophins-mediated signalling and synaptic activity.

Most research in this expanding field is conducted on the hippocampus, due to the well-known involvement of this structure in long-term potentiation and memory. For the first time this thesis reports the overexpression of a cluster of
genes involved in neurodevelopment in the PrL, another brain structure that is crucial for cognitive processes, attention and working memory. This finding is in accordance with the association between neurotrophic factors expression and cognition-relevant synaptic activity that is reported in the literature (Lu et al., 2003).

The complexity of the biological mechanisms that modulate higher cognitive functions is likely to require the involvement of multiple factors. In this scenario, microarray analysis could be exceptionally beneficial, as it allows the simultaneous investigation of many genes. In fact, this strategy led to the discovery that the PrL overexpress both BDNF and synapsin II, two genes that have a role in the same biological pathway modulating synaptic transmission and long-term potentiation (Jovanovic et al., 2000).

The role of other neurodevelopment-related genes overexpressed in the PrL (table 3.3) is less clear. The overexpression of genes such as DPYS1.3, DPYS1.5, doublecortin and also BDNF suggests that the PrL neurons may develop novel synaptic connections more frequently than the VO and the M1, and that this highly dynamic organisation of the PrL neurocircuitry could underlie the specific functions of this subregion in cognitive and attentional processes. However, functional studies would be necessary to investigate this hypothesis.

The finding that the PrL overexpresses neurodevelopment-related genes also implies that this expression pattern could be considered as a molecular fingerprint of areas involved in certain cognitive processes, especially memory. This is supported by the well-known expression of neurotrophins such as BDNF in the hippocampus, and their role in long-term potentiation and memory formation (Lu et al., 2003). The amygdala is another structure where neurotrophins are known to regulate the synaptic plasticity underlying fear conditioning and therefore amygdala-dependent learning and memory (Rattiner et al., 2005).

Although molecular characterisation of amygdaloid nuclei and hippocampal regions using microarrays has been performed (Zirlinger et al., 2000; Bonaventure et al., 2002; Zirlinger, 2003; Datson et al., 2004; Torres-Munoz et al., 2004), it is difficult to compare the expression profiles of neurodevelopment-related genes
reported in these studies to the present work for multiple reasons. First, different research strategy, such as the choice of controls, can make direct comparison of expression profiles misleading. Second, comparison of microarray data obtained using different platforms can lead to unreliable conclusions due to the notorious lack of reproducibility across different arrays. However, results from the present study and evidence from the literature strongly suggest that the expression of neurodevelopment-related genes in specific brain regions is a landmark of a high degree of synaptic adaptability to external stimuli and potentially underlies cognitive and mnemonic processes.

The increased popularity of microarray analysis in neuroscience and recent technological advances have rapidly improved the reproducibility of results when the same platform is used. Therefore, the possibility of investigating the expression of this class of genes in other brain structures and cortical subregions can contribute to identifying the neurobiological basis of cognition.

3.4.3 Localised expression of genes involved in neurotransmission

The LMD/microarray analysis showed that each of the brain regions examined overexpresses a number of genes involved in neurotransmission. The genes that were classified as neurotransmission-related according to their gene ontology have a well-known function that is crucial to synaptic transmission; such mRNAs encoded for enzymes controlling the synthesis of neurotransmitters, membrane receptors and effectors.

Consistent with the prevalence of GABAergic inhibitory neurons in the Rt, this region overexpressed the two isoforms of glutamate decarboxylase GAD65 and GAD67, which control the biosynthesis of GABA (table 3.2, "Neurotransmission" functional class).
The glutamate ionotropic AMPA receptor subunit GluR4 was also overexpressed in the Rt (table 3.2, “Neurotransmission” functional class); this finding was strongly significant as the False Discovery Rate was below 0.1% when the expression values recorded in the Rt were compared to the Prl, VO and MI. Moreover, the Fold Change (FC) associated with the comparison between the Rt and the other regions ranged from 2.5 to 3.1; these values are remarkably high, as expression changes in the brain are usually very small.

These findings suggest that GluR4 may have a crucial role in the stimulation of the inhibitory interneurons of the Rt. This brain region receives inputs from excitatory collateral fibres generating from the cortico-thalamic and thalamo-cortical projections (Paxinos, 1995). It has been shown that in the rat the Rt also receives inputs from the brain stem and the basal forebrain, and that the majority of these fibres are cholinergic (Hallenger et al., 1987; Asanuma, 1989). The abundance of AMPA receptor GluR4 subunit in the Rt suggests that the stimulation exerted by the glutamatergic fibres originating from the cortico-thalamic and thalamo-cortical projections could be predominant over the cholinergic inputs for the activation of this thalamic nucleus.

The δ isoform of the protein kinase C (PKCδ), a crucial enzyme for the transduction of signal mediated by protein Gq-coupled receptors (see chapter 4), was also overexpressed in the Rt. It is difficult to predict the functional implications of this finding, as PKCδ activity can have multiple biological functions (see chapter 4); however, the observation that this gene is highly overexpressed in the Rt (Fold Change ranging from 3.1 to 4.7) suggest that PKCδ may play a crucial role in the neurobiology of this brain region.

Another interesting finding is the localised overexpression of specific GABA_A receptor subunits. The LMD/microarray study showed that the β1 and the α5 subunits are overexpressed in the Prl, whereas the δ subunit is overexpressed in the VO. Several studies analyse the regional distribution of GABA_A receptor subunits in the rat brain both at the mRNA level and at the protein level, analysing the expression levels in major brain areas, such as neocortex, thalamic and hypothalamic nuclei, and cerebellum (Benke et al., 1991; Wisden et al., 1992;
Fritschy et al., 1995; Pirker et al., 2000). The subunit composition of GABA\(_A\) receptors within a specific brain region has a great biological importance, as it determines the physiological characteristics of the inhibitory synapses, as well as their pharmacological properties (Fritschy et al., 1995). Some publications highlight that the specificity of benzodiazepines for GABA\(_A\) receptors depends on their subunit composition (Pirker et al., 2000). For the first time the present study clearly shows that not only the GABA\(_A\) receptors subunits composition is specific to the brain areas, but also, at a higher level of anatomical specificity, subregions of the prefrontal cortex such as the PrL and the VO show overexpression of specific subunits. This finding highlights that the GABA\(_A\) receptors subunits composition can be analysed with a greater degree of anatomical specificity. It also suggests that the specific subunits overexpressed in the PrL and the VO may contribute to the electrophysiological and neurobiological characteristics that underlie the cognitive functions of these subregions.

### 3.4.4 Localised expression of genes involved in ion transport

Another interesting finding of the LMD/microarray study is the localised expression of genes involved in the control of ion transport. Four different potassium channels were overexpressed in the Rt. The potassium channels overexpressed in this area have different structure and biological properties.

One of the genes encoded for the Kv3.1 potassium channel, a member of the Kv3 subfamily. The Kv3 potassium channels allow the efficient re-polarization of the neuronal membrane after high-frequency repetitive firing (Rudy et al., 2001) and therefore are expressed in neuronal population that fire action potentials with high frequencies (Gan et al., 1998). Weiser et al. (1995) reported that the Kv3 potassium channels are expressed in the ventral thalamus, including the Rt; moreover this subunit is expressed in parvalbumin-containing interneurons. The
LCM/microarray study showed that the Rt overexpresses both Kv3 potassium channels and parvalbumin, in accordance with the prevalence of GABAergic inhibitory neurons in this brain region. This finding suggests that Kv3 channels and parvalbumin (see chapter 4) could contribute to the specific neurobiological properties of the Rt inhibitory neurons.

Kv11.1 is another potassium channel that was overexpressed in the Rt. This voltage-gated, six transmembrane domains channels belongs to the EAG-related subfamily, which was first described in Drosophila (reviewed by Bauer et al., 2001). It is difficult to predict the biological function of Kv11.1 in the Rt; however its overexpression in this area suggest that it may play a specific role in the ion trafficking in neurons of this area, contributing to the neurotransmission.

FXYD6 was the gene involved in ion transport that showed the most specific regional expression pattern. This gene, which is involved in the modulation of the Na-K-ATPase activity, was overexpressed in the PrL with a Fold Change ranging from 9.35 to 10.21; suggesting that ion trafficking is regulated in a region specific manner (see chapter 4).
CHAPTER 4. CONFIRMATION OF REGIONAL
EXPRESSION PROFILES GENERATED BY
LMD/MICROARRAY USING IN SITU
HYBRIDISATION
4.1 Introduction

4.1.1 Confirmation of microarray results

Standardised manufacturing processes and increased use in gene expression analysis have made microarray technology increasingly more reliable and robust. Moreover, the recent statistical packages for microarray data analysis allow a strict control over the false discovery rate (the FDR is a convenient method to express statistical significance of microarray data. The FDR value indicates the proportion of false positives in a certain set of tests declared positive by statistics; see chapter 3). However, a number of false positive results could still be detected, due to the amount of data generated by this type of experiment. In this scenario, confirmation of differentially expressed genes by alternative techniques is mandatory to validate the microarray results and to verify the reliability of crucial upstream manipulations, such as linear amplification.

In situ hybridisation (ISH) was selected as the technique of choice for the validation of the present microarray study. As the microarray experiment was designed to identify localised gene expression patterns, ISH was an obvious choice for the confirmation of this type of data. In fact, this technique has the advantage of visualizing the transcript in the anatomical structure where it was expressed; moreover, it is suitable for accurate quantification of the expression levels (Wisden and Morris, 2002). The ISH autoradiograms also allow the analysis of the laminar expression in the cortical layers; this additional information is very useful to complement the data obtained with the LMD/microarray strategy. Another advantage of ISH is that this technique does not involve any amplification of the amount of mRNA originally expressed in the anatomical structure analysed; therefore it is helpful to verify the validity not only
of the microarray hybridisation, scanning and analysis processes, but also the linearity of the amplification procedure.

Clearly, a comprehensive validation of the vast amount of data generated by a microarray study is not practically achievable; therefore, verification experiments can be performed only on a small number of selected genes. For the present study, 13 genes were selected for validation using ISH (table 4.1). The selection criteria were based on the localised differential expression and the function of the genes. Firstly, genes enriched in all schizophrenia related regions examined in the microarray study (PrL, VO and Rt) were selected for validation. Additionally, the validation experiments included genes that showed False Discovery Rate ranging from 0.00 to 0.93 and Fold Change ranging from 1.51 to 17.77 in order to verify both small and great expression differences. Lastly, the biological function of the genes was taken into account, giving priority to genes that could be involved in the neuropathology of schizophrenia and genes controlling crucial neurobiological mechanisms in the cortex and the Rt, such as neurotransmission and development.
### Regional selectivity

<table>
<thead>
<tr>
<th>Gene</th>
<th>45mer probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXYD6</td>
<td>CTC AGA GCC AAG AAT TCC TGG GTG</td>
</tr>
<tr>
<td></td>
<td>TTC TGT AAA GGG TGG GGC GGA</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;α&lt;/sub&gt;31</td>
<td>AAG AAC ATT CCG GAC CAC TTG TCT ATG</td>
</tr>
<tr>
<td></td>
<td>GAG TTC ACG TCA GTC AAG</td>
</tr>
<tr>
<td>PrL</td>
<td>GCC CTC GTG TAT CTG AAG AGC TAA TCT</td>
</tr>
<tr>
<td></td>
<td>GAA AAG AGG CTG GCA AAG</td>
</tr>
<tr>
<td>Synapsin II</td>
<td>CCT CAC AGC AAC ATG CAC CGA GGC</td>
</tr>
<tr>
<td></td>
<td>GTC CTC AGG TGG CAG ACA CTC</td>
</tr>
<tr>
<td>Wolframin</td>
<td>TCT GGC CCA GAG GTT AAA AAT AGA TCT</td>
</tr>
<tr>
<td></td>
<td>CCT ACC ACA ATT TCT ATC</td>
</tr>
<tr>
<td>Doublecortin</td>
<td>GCC GTC GCC GTA GGG GCT CAG CTT</td>
</tr>
<tr>
<td></td>
<td>CTC GTC GCC GTA GGG CAG CAC</td>
</tr>
<tr>
<td>CaMKII inhibitor α</td>
<td>ATG GCG TTC CTC ACG TCC ATC TCT</td>
</tr>
<tr>
<td>VO</td>
<td>GCC CTT GCC TCC AGG TCC ACG TCT</td>
</tr>
<tr>
<td></td>
<td>CTC GCC GTA CAC ATA ATT</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;δ&lt;/sub&gt;</td>
<td>AGC CAG CAG GTT GGG AGA ACA GCT</td>
</tr>
<tr>
<td></td>
<td>CCC ACA GAG TGG AAG AAG TTG</td>
</tr>
<tr>
<td>Stk23</td>
<td>TTC TAA GTT AAG AAA ACT GAC AGG GTG</td>
</tr>
<tr>
<td></td>
<td>AGG GCT CTC CTA TTA CCG</td>
</tr>
<tr>
<td>RGS3</td>
<td>TGT GCC ATA CAT TGA CTT AAG CGG TAT</td>
</tr>
<tr>
<td></td>
<td>TTA TTT GGT GTT AGG TTA</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;δ&lt;/sub&gt;4</td>
<td>TTC TTC ATG GGC AGT GCG GAT GCA</td>
</tr>
<tr>
<td></td>
<td>AAC CAG GGC AGA TGG GCC TAC</td>
</tr>
<tr>
<td>PKC&lt;sub&gt;δ&lt;/sub&gt;</td>
<td>CCC CAG CTC ATC CTC CTC AAT GAA</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>GCC ACT TTT GTC TTT GTC CAG</td>
</tr>
<tr>
<td>Calretinin</td>
<td>GAT ATT CAT CTC CTT GCT TGT GTC CTC</td>
</tr>
<tr>
<td></td>
<td>ATA CAG ATC CTT CAG GAG</td>
</tr>
</tbody>
</table>

### Table 4.1

Genes showing region-selective expression according to the LMD/microarray study that were selected for validation by in situ hybridisation. (Refer to chapter 2 for details on design of the specific 45mer oligonucleotide probes).
4.1.2 Genes overexpressed in the Prl.

4.1.2.1 FXFD6

The FXFD family is composed of small hydrophobic proteins with a single transmembrane domain (Cornelius et al., 2003). This group of short proteins is named after their conserved four aminoacids motif, and are thought to regulate the activity of the Na-K-ATPase (Geering, 2006; Garty et al., 2005).

The Na-K-ATPase is a ubiquitous enzyme that is crucial to actively maintain the physiological ionic concentration of the intracellular compartment. Some important biological roles of this transporter are the restoration of the resting membrane potential in excitable cells and providing energy for co- and counter-transport systems (Geering, 2005). Specific cells finely modulate the activity of Na-K-ATPase according to their biological characteristic using different mechanisms, such as phosphorylation, expression of specific isoforms of the enzyme and expression of specific modulator proteins (Geering, 2005).

The members of the FXFD family are thought to be tissue-specific regulators of the Na-K-ATPase and are preferentially expressed in electrically excitable cells, which require a complex and dynamic modulation of the enzyme (Crambert et al., 2002; Beguin et al., 2002; Geering et al., 2003). These proteins directly interact with the Na-K-ATPase via hydrophobic interactions and modify the enzyme kinetics (Cornelius et al., 2003).

FXFD6, also known as phosphohippolin, has been recently characterised and cloned (Kadowaki et al., 2004). Protein expression studies in rat show that FXFD6 is predominantly present in the brain, and in vitro evidence suggests that the protein is present in neuronal cells, but not in glial cells (Kadowaki et al., 2004). Interestingly, the expression level of FXFD6 in the forebrain, hippocampus, cerebellum and brain stem changes during postnatal development, decreasing after 3 weeks (Kadowaki et al., 2004).
The role of FXYD6 suggests that this regulator protein could have a crucial role in the ion transport and neurotransmission in neurons of specific brain areas; moreover, the development related expression changes reported by Kadowaki et al., (2004) suggest that this protein may have a role in neurodevelopment.

4.1.2.2 Wolframin

The wolframin gene encodes an 890-aminoacid protein that does not show homology with any known sequence (Inoue et al., 1998). Although the structure of this macromolecule is currently unknown, prediction suggests that it presents nine or ten transmembrane domains (Strom et al., 1998; Hofmann et al., 2003). Recent studies show that wolframin is predominantly localised in the endoplasmic reticulum (Cryns et al., 2003; Osman et al., 2003), and that it is likely to be an integral endoglycosidase membrane glycoprotein (Takeda et al., 2001).

The exact biological function of wolframin is currently unclear; however, Osman et al (2003) showed that it is involved in calcium transport in the endoplasmic reticulum. In fact, overexpression of wolframin in oocytes resulted in increased intracellular calcium concentration, as a consequence of an increased release of this ion from the endoplasmic reticulum (Osman et al., 2003).

Following these findings, it was suggested that wolframin might be a novel calcium channel located in the endoplasmic reticulum, or alternatively, a regulator of calcium channel activity in this subcellular location. This function may be crucial for excitable cells, such as neurons, which require a rapid calcium transfer from the endoplasmic reticulum for ion homeostasis and regulation of secretion.

Most of the research on wolframin focuses on the role of this gene in Wolfram syndrome. This autosomal recessive neurological disease, also known as DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy and deafness) is caused by mutations on the wolframin gene, suggesting that loss of function of the wolframin protein leads to the neuropathological changes underlying the complex symptomatology of Wolfram syndrome (Gerbitz et al., 1999). Patients suffer from
non-inflammatory atrophic changes in pancreatic islets, optic nerve, chiasm, tracts and a number of brain structures such as lateral geniculate nuclei, basis pontis, hypothalamic periventricular and supraoptic nuclei (Scolding et al., 1996; Shannon et al., 1999; Khanim et al., 2001). These widespread degenerative changes trigger the onset of diabetes, vision loss and seroneural deafness.

Interestingly, Wolfram syndrome patients show a high proportion of hospitalisation for psychiatric illness and self-reported mental illness (Swift et al., 2001). Although the role of wolframin in psychosis remains unclear, evidence show that mutations of this gene can be associated with at least some forms of psychiatric illness (see chapter 5).

4.1.2.3 Synapsin II

Synapsin II belongs to a family of proteins specifically expressed by neurons that are associated with the membrane of the synaptic vesicles (reviewed by Hilfiker et al., 1999; Ferreira et al., 2002). Synapsins are encoded by three different genes, synapsin I, II and III; additionally, alternative splicing of the transcripts yields the isoforms Ia, Ib, IIA and IIb (Kao et al., 1999).

The structure of synapsin isoforms includes variable domains, however some domains that are crucial for their subcellular localisation and function are conserved in all isoforms (Sudhof et al., 1989). The domain A is present in all synapsins, and it forms the substrate for phosphorylation catalysed by both protein kinase A and Ca^{2+}/calmodulin dependent kinase I (CaMKI) (Hosaka et al., 1999). This site is crucial for the association of the protein with the membrane of the synaptic vesicle; a molecular interaction that is finely regulated by phosphorylation (Hosaka et al., 1999). The domain C is also conserved; this structure is a binding site for ATP (Sudhof et al., 1989). Although synapsins do not show constituent ATPase activity, it has been suggested that synapsins may have an enzymatic role using the ATP hydrolysis to catalyse some unidentified...
reaction (Hilfiker et al., 1999). This hypothesis is supported by the structural similarity between the domain C and some bacterial ATP-dependent synthetases (Esser et al., 1998). Another important function of the domain C is the binding with other synapsins, which mediates the formation of homo- or heterodimers (Hosaka et al., 1999).

The role of synapsins is closely related to their subcellular localization. Their association with the synaptic vesicles suggests that these proteins regulate neurotransmitter release. In fact it has been demonstrated that synapsins regulate the availability of synaptic vesicles for exocytosis (Hilfiker et al., 1999). In the presynaptic terminals only a small number of vesicles, named the releasable pool, are located in the proximity of the plasma membrane and are available for immediate release. The reserve pool, which comprises the majority of vesicles available in the presynaptic terminal, is located away from the plasma membrane (Greengard et al., 1993). This organisation allows fine modulation of neurotransmission and important biological mechanisms such as synaptic plasticity. Synapsins play a central role in the organisation of presynaptic terminals, as they regulate the number of vesicles held in the reserve pool. The synapsin-held vesicles are required for neurotransmission during high levels of neuronal activity, such as upon high frequency stimulation that elicits synaptic depression (Hilfiker et al., 1999).

Regulation of the proportion of vesicles held in the reserve pools is not the only mechanism by which synapsins modulate neurotransmission. Domain E, which is found in the Ia, Ila and III isoforms (Kao et al., 1998; Sudhof et al., 1989), mediates control over neurotransmitter release kinetics. It has been suggested that domain E-containing synapsins directly slow the reaction of fusion between the vesicles and the plasma membrane, an event that is independent from the distribution of the vesicles in the releasable and reserve pools (Hilfiker et al., 1999).

Recent studies focus on additional functions of synapsins (reviewed by Ferreira et al., 2002). Along with the complex control over kinetics of neurotransmitter release, these proteins play a key role in synapse development
and maintenance. Specifically, it has been found that suppression of synapsin II impairs synaptogenesis in developing hippocampal neurons and causes synaptic loss in neurons that had formed their synapses (Ferreira et al., 1995). Moreover, synapsin II-depleted hippocampal neurons show reduced and aberrant neurite elongation (Ferreira et al., 2002).

The multiple and complex roles of synapsin II in neurotransmission and development make the regional expression profile of this gene interesting and relevant to pathologies that may involve alteration of synaptic transmission. In fact, recent studies report the involvement of synapsin II in schizophrenia and in the neurochemical changes following antipsychotic administration (see chapter 5).

4.1.2.4 GABA_\textsubscript{A}β1

GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the mammalian brain. This molecule binds to three types of membrane receptors, classified as GABA\textsubscript{A}, GABA\textsubscript{B} and GABA\textsubscript{C}. The GABA\textsubscript{A} receptors are transmembrane ion channels with selective permeability to chloride ions that become activated when GABA binds to a specific domain on the extracellular side. These GABA-activated channels mediate fast inhibitory neurotransmission by allowing an influx of chloride ions in the postsynaptic neuron, and therefore hyperpolarizing the postsynaptic membrane.

GABA\textsubscript{A} receptors have a pentameric structure, which is assembled with subunits from at least 19 types classified according to their amino acid sequence homology in the following groups: α, β, γ, δ, ε, π, θ, and ρ (Rabow et al., 1995; Bonnert et al., 1999). Although all the subunit types could theoretically be assembled in thousand of different pentamers, the majority of the GABA\textsubscript{A} receptors present a fixed combination of two α, two β and one γ subunits (Rabow et al., 1995).
The exact subunit composition of the GABA<sub>A</sub> receptor determines its neurochemical and pharmacological properties (reviewed by Mehta et al., 1999). The affinity for the ligand and the pharmacological specificity for allosteric modulators such as benzodiazepines are two key features that are strongly influenced by the presence of specific subunits (Bureau et al., 1990; Pritchett et al., 1990; Pritchett et al., 1989).

The importance of the GABA<sub>A</sub> subunit composition is supported by their highly diversified expression across the brain (Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000), suggesting that differential expression of the genes encoding for these proteins is crucial to the neurochemistry of specific brain regions (Steiger et al., 2004). Moreover, GABA<sub>A</sub> subunit expression shows clear changes during post-natal development, probably reflecting changes in the neuronal composition and neurocircuitry (Laurie et al., 1992).

β1 is one of the subunits that show very specific regional expression in the rat brain. Wisden et al. (1992) reported that the mRNA encoding for the β1 subunit is abundant in the hippocampus, with lower levels of expression in the amygdala, neocortex, basal nuclei and septum. The same study reported a higher expression in the deep layers of the neocortex compared to layers 1, 2 and 3. Interestingly, the expression pattern of the β1 subunit is similar to that of the α5 subunit (Wisden et al., 1992), suggesting that the proteins may colocalise and that GABA<sub>A</sub> receptors containing them may have a role in the physiology and neurochemistry of areas such as the hippocampus and the neocortex.

Although the overall expression pattern of the GABA<sub>A</sub> receptor β1 subunit has been reported both at the mRNA level and at the protein level (Pirker et al., 2000; Wisden et al., 1992), there is no quantitative study analysing the expression differences of this gene in specific cortical subregions.
4.1.3 Genes overexpressed in the VO

4.1.3.1 GABA_δδ

Studies based on immunocytochemistry and in situ hybridisation show that the δ subunit of the GABA_δ receptor is expressed in the cerebellum, thalamus, hippocampus, neocortex and striatum (Pirker et al., 2000; Wisden et al., 1992).

Pirker et al. (2000) also showed that the distributions of the γ and the δ subunits are complementary, suggesting that these subunits do not co-localise, and that GABA_δδ could be incorporated in the pentameric complex instead of GABA_δγ with some specific α subunits. In fact GABA_δδ co-localise with the α4 subunit in the thalamus and with the α6 subunit in the cerebellum (Pirker et al., 2000). Although it has been shown that α4 and α6 can form functional complexes with the γ subunit as well, their specific co-localisation with the δ subunit suggests that these receptors may have a specific function in the brain regions where they are expressed. A study analysing the subcellular distribution of GABA_δδ-α6 complexes in the cerebellar granular cells showed that these receptors are exclusively localised in the extrasynaptic dendritic and somatic membranes, where they mediate tonic inhibition (Nusser et al., 1998).

The overall distribution of GABA_δδ in the brain cortex has been investigated both at the mRNA and at the protein level (Pirker et al., 2000; Wisden et al., 1992); however there is no quantitative study analysing the expression differences of this gene in specific cortical subregions.

4.1.3.2 CaMKII inhibitor α

CaMKII (Calcium/calmodulin-dependent kinase) is a serine/threonin kinase activated by the Ca\(^{2+}\)/calmodulin complex (reviews by Hudmon et al., 2002;
Yamauchi, 2005). This enzyme phosphorylates a broad range of substrates, including structural proteins, enzymes and important regulatory protein in the brain such as synapsins (Bennett et al., 1983). Depending on the site of expression and the substrates, CaMKII modulates many biological pathways in response to increased intracellular calcium concentration. CaMKII catalysed phosphorylation is known to regulate cytoskeletal organisation, intracellular calcium homeostasis, carbohydrate metabolism and neurotransmission (Hudmon et al., 2002). Although CaMKII is ubiquitous, the expression in the brain is particularly prominent, suggesting that this enzyme is crucial for the modulation of important neuronal functions and to coordinate the cellular response to Ca^{2+}, which is the most common second messenger.

One of the roles of CaMKII in the brain is the modulation of learning and memory. Strong evidence support the role of this enzyme in cognition; in fact its activity is necessary for long term potentiation, a form of synaptic plasticity that is crucial for learning processes (Malinow et al., 1989; Fukunaga et al., 1993; Otmakhov et al., 1997; Ouyang et al., 1997; Hinds et al., 1998).

Many features of CaMKII and its involvement in complex cortical functions depend on the regulation of the enzymatic activity. In absence of stimulus, CaMKII is inhibited by an autoregulatory domain, which binds the catalytic domain preventing interaction with the substrate. Both catalytic and autoregulatory domains are located on the same polypeptide (Kemp et al., 1996). The bond with the Ca^{2+}/calmodulin complex disrupts the interaction between the catalytic and the autoregulatory domain, exposing the site of interaction with the substrate and therefore activating the enzyme (Hook et al., 2001).

Along with the activation mechanism from basal state described above, the activity of CaMKII can be finely modulated by regulatory autophosphorylation, which disables the autoregulatory domain causing a Ca^{2+}/calmodulin independent activation (Hudmon et al., 2002). Moreover, CaMKII can be inhibited by the recently characterised specific inhibitor proteins (Chang et al., 1998; Chang et al., 2001).
Two isoforms of CaMKII inhibitor have been characterised and named CaMKIIα and CaMKIIβ, as they co-localise with the α and β isoforms of the enzyme respectively. CaMKII binds the catalytic domain of the enzyme causing a potent non-competitive inhibition. Although CaMKII is widely expressed in many types of cells, the expression of the inhibitor is much more restricted; interestingly, CaMKIIα seems to be specific for the brain (Chang et al., 1998; Chang et al., 2001). These findings suggest that along with the Ca²⁺/calmodulin interaction and the autophosphorylation, CaMKII activity can be regulated at a further level in the brain. The tissue specific expression of CaMKII may be crucial to the fine enzymatic modulation that underlies synaptic plasticity and therefore cognitive processes.

The regional distribution of CaMKIIα mRNA in the rat brain has been described by Chang et al. (2001). ISH analysis showed that the transcript is present predominantly in the forebrain and the hippocampus, with little or no signal in the midbrain and in the cerebellum. The same study reported expression also in the olfactory bulb and the caudate-putamen; moreover, the mRNA was more prominent in the layers II and III of the neocortex compared to the deep layers.

4.1.4 Genes overexpressed in the Rt

4.1.4.1 RGS3

Guanine nucleotide-binding proteins (G protein) are ubiquitous effectors that are coupled to a vast number of membrane receptors. In the brain G proteins play a key role in the signal transduction of neurotransmitters binding specific receptors with seven transmembrane domains that are coupled with this mechanism (Manji, 1992).
When inactive, G proteins are a heterotrimeric complex constituted by the subunits α, β and γ associated to the seven membrane-spanning segments receptor. The Ga subunit contains the binding site for the guanine nucleotide, and is associated to GDP in the inactive state. The GDP stabilises the interaction with the β and γ subunits, maintaining the transduction system inactive when the receptor is not activated. Binding of the ligand on the specific site of G protein-coupled receptors elicits a conformational change that is transmitted to the intracellular side; consequently, the Ga subunit releases GDP and binds GTP. The guanine nucleotide switch is a key step in the activation/inactivation cycle of G protein mediated signal transduction, as the Ga subunit loses affinity for the βγ complex when it is bound to GTP. The dissociated Ga-GTP complex is the activated factor that interacts with specific effector enzymes stimulating their activity. Inactivation occurs when the GTP is hydrolysed to GDP, restoring the affinity of the Ga subunit for the βγ complex; the following re-formation of the heterotrimeric complex completes the cycle.

The Ga subunit has an intrinsic GTPase activity that is crucial for the termination of the signal. However, the rate of GTP hydrolysis in vitro does not correspond with the faster kinetics shown by the signal termination in vivo (Drats et al., 1987; Vuong et al., 1991), suggesting that other mechanisms contribute to the regulation of GTP hydrolysis in the G protein activation/inactivation cycle.

In fact, the GTPase activity of the Ga subunit is enhanced by a group of proteins known as regulators of G protein signalling (RGS) (reviewed by Dohlman et al., 1997; Ross et al., 2000) RGS interact directly with the Ga subunit and increase the rate at which the GTP is hydrolysed to GDP (Ross et al., 2000). Interestingly, RGS do not make direct contact with the GTP, and therefore are not likely to interact with the catalytic GTPase site. This suggests that RGS do not have a direct role in the chemistry of GTP hydrolysis, but rather cause a conformational change in the Ga subunit structure that increase the efficiency of the GTPase (Tesmer et al., 1997; Sprang, 1997).

The activity of RGS is crucial in the brain, as they determine the kinetics of termination of the signalling initiated by a number of neurotransmitters.
Currently, more than 20 different mammalian RGS proteins have been identified (Ross et al., 2000), and little is known about the exact physiological role of specific isoforms. Also, it seems unlikely that each RGS protein regulates one, or a small subset of Ga subunits (Dohlman et al., 1997; Bourne et al., 1991; Mittal et al., 1996); therefore it is difficult to understand the biological significance of the diversity of RGS proteins.

A degree of selectivity is given by the specific expression of RGS proteins. As expected, most of the RGS proteins are found in the brain, where fine regulation of signal transduction is paramount; however the expression of specific RGS is known to be restricted to anatomical structures (Gold et al., 1997). Gold and co-workers showed that RGS3 is one of the isoforms with the most restricted expression pattern. No mRNA encoding for this protein was found in the cortex, except for very faint signal in the piriform cortex; expression was also low or absent in the brainstem and hypothalamus. The only site that showed prominent expression of RGS3 according to Gold’s analysis was the thalamus.

4.1.4.2 PLC\(\beta\)

PLC\(\beta\)s (phospholipase C \(\beta\)) are a crucial component of the Gq protein signalling pathway. The enzymes belonging to the PLC family degrade phosphatidylinositol-4,5 bisphosphate (PIP\(_2\)) to produce diacylglycerol (DAG) and inositol-1,4,5 trisphosphate (IP\(_3\)) (reviewed by Rhee, 2001). DAG and IP\(_3\) are second messengers with important functions, such as the activation of PKCs (see above) and the mobilization of Ca\(^{2+}\) ions from the intracellular pool.

The enzymes belonging to the different PLC subclasses (\(\beta, \gamma, \delta\) and \(\varepsilon\)) do not have conserved sequences, except for the catalytic domains, known as the X and Y domains. The PH (pleckstrin homology) domain is present in the PLC \(\beta, \gamma, \delta\), and \(\varepsilon\), although the aminoacidic sequence is partly specific; the differences in the PH domain contribute to the different mechanisms of activation of the isozymes (Litosch, 2002). The main structural difference between PLC\(\beta\)s and the isozymes
of the other subclasses is the presence of a long sequence of approximately 450 amino acids at the C terminal. This domain has several key functions, such as the subcellular localization of PLCβs to membranes or cell nucleus (Kim et al., 1996) and the specific activation of this subclass by G proteins (Wu et al., 1992).

The main mechanism of activation for PLCβs is via the interaction with protein Gaq-GTP. The protein Gaq is activated by the interaction of a seven transmembrane domains receptor with its specific ligand. This event is followed by the release of GDP from the Gaq subunit, which consequently binds GTP and dissociates from the βγ subunit. The activated Gaq-GTP complex interacts with the C-terminal domain of PLCβs, causing the activation of the enzyme.

Although all the PLCβs are activated by Gaq, there are other mechanisms of activation that can be specific for some isoforms of the enzyme. Currently, four isozymes are known, namely PLCβ1, PLCβ2, PLCβ3 and PLCβ4 (Rhee, 2001). An alternative mechanism of activation is via the interaction with the βγ subunit of G proteins after it is released from the α subunit (Smrcka et al., 1993; Lee et al., 1994). The βγ subunit is common to all the G proteins; therefore PLCβs can be activated not only by Gaq proteins, but also by the βγ subunit released from the Gas and Gai proteins. PLCβ4 is the only isozyme that is not activated by the βγ subunit; hence it specifically requires Gaq interaction for its activity (Rhee, 2001).

The PLCβ isozymes differ not only for the mechanism of activation, but also for their specific expression pattern. PLCβ4 expression is restricted to the retina and some brain regions. Roustan et al. (1995) reported that this isoform is abundant in the cerebellar Purkinje cells and is also present in the substantia nigra, the medial geniculate bodies and the thalamic nuclei.

4.1.4.3 PKCθ

The PKCθs (protein kinase C) are a group of serine/threonine kinases that are involved in a vast number of biological functions, including cell growth and
apoptosis. The specific role of each isoform of the PKC family depends on the subcellular localization as well as on structural characteristics of the enzyme (reviewed by Steinberg, 2004).

The PKCs are single polypeptide chains, with a highly conserved C-terminal catalytic domain; this sequence contains the binding sites for the ATP and the substrate. The N-terminal contains the autoregulatory pseudosubstrate domain and the membrane-targeting domains, named C1 and C2. The function of the C1 and C2 domains is to determine the subcellular localization of the enzyme, which is usually associated with the plasma membrane (Cho, 2001; Hurley et al., 2000). The N-terminal structure is not conserved, but varies in different isoforms and determines the mechanism of activation of the enzyme (Steinberg, 2004); in fact the PKCs are subdivided into three main families according to the structure of their regulatory domains.

The cPKCs (conventional PKCs) contain both C1 and C2 membrane targeting modules. The C1 domain is the binding site for DAG (diacylglycerol), which is crucial for the activation of the enzyme and for its translocation to the plasma membrane. The C2 domain binds anionic phospholipids on membranes in a calcium dependent manner, as this module contains multiple calcium binding residues. The cPKCs are therefore activated by DAG and Ca^{2+}, which are the second messengers released after activation of receptors coupled with protein Gq.

The nPKCs (novel PKCs), also have C1 and C2 domains; however, the C2 module lacks the calcium binding motifs. Hence the nPKCs are activated mainly by DAG, and do not require increase of intracellular calcium concentration. PKCθ belongs to this subclass; this isoform has a unique C2 domain containing a binding site for SFK (Src family kinase), therefore the phosphorylation of the C2 domain is a specific regulatory mechanism for PKCθ (Pappa et al., 1998)

Another class of PKCs is the atypical isoforms (aPKCs), which lack a C2 domain and do not bind DAG, therefore the mechanism of activation of these enzyme is DAG/Ca^{2+} independent, and involves different phospholipid cofactors (Hirai et al., 2003; Farese, 2002).
The activation of PKCδ is mainly dependent on the binding of DAG and the consequent translocation of the enzyme to the plasma membrane. The interaction between the DAG-C1 domain and the membrane cause a conformational change that frees the catalytic domain from the autoregulatory pseudosubstrate domain, activating the enzyme (Steinberg, 2004). Phosphorylation of an activation loop is also an important mechanism of activation for PKCδ, as suggested by recent studies (Hamaguchi et al., 2003; Rybin et al., 2003; Rybin et al., 2004).

The PKCs have multiple roles and participate in several complex biological pathways; their subcellular localisation seems crucial not only for the activation but also for the substrate specificity. It has been shown that PKCδ is involved in the biological pathways controlling cell cycle and apoptosis (Steinberg, 2004). Unlike other isoforms, PKCδ slows proliferation and promotes differentiation (Fukumoto et al., 1997; Ashton et al., 1999; Braun et al., 2003). PKCδ also has a role in the modulation of inflammatory processes, as it activates NFκB, which is a transcription factor controlling several biological processes including inflammation and immune response (Page et al., 2003). The exact biological function of PKCδ in the brain and specifically in the Rt is unclear. However, the overexpression of the mRNA encoding for this enzyme in the Rt could have a relevant biological significance, as it is involved in the modulation of crucial biological pathways, such as the activation of the MAPK (mitogen-activated protein kinases) cascade and the response to neurotransmitters activating Gq proteins (Steinberg, 2004).

4.1.4.4 Parvalbumin

Parvalbumin is a low molecular weight protein that binds Ca\(^{2+}\) ions with high affinity. The structure and the chemical properties of parvalbumin have been described by several authors (reviewed by Pauls et al., 1996). X-ray crystallography and NMR studies show that the parvalbumin polypeptide is
folded into six helices, named A to F, that form three “EF hands” domains. These structures, composed by two helices spaced by a loop, are the binding sites for the Ca^{2+} ions (Pauls et al., 1996); in fact, the metal ions interact with seven residues situated in the Ca^{2+} binding loop (Strynadka et al., 1989). Only two of the parvalbumin EF hands are functional, hence this protein can bind two Ca^{2+} ions. In the third EF hand, the two helices are connected by a non-functional truncated loop, which is unable to interact with metal ions (Pauls et al., 1996).

Unlike many Ca^{2+}-binding proteins that feature EF hands (see below), parvalbumin metal-binding sites are not specific to calcium. In fact, the parvalbumin EF hands are best described as Ca^{2+}/Mg^{2+} mixed metal-binding sites with high affinity for calcium and moderate affinity for magnesium, where the two metal ions compete for the same binding site (Haiech et al., 1979). The different affinity for calcium and magnesium is a key feature of parvalbumin and influence the buffering capacity of this protein, as the selectivity for the two metal ions depends on their relative concentration (Pauls et al., 1996).

Although the structure and the biophysical properties of parvalbumin are very well known, its physiological role is still unclear, especially in non muscular cells. Parvalbumin is abundant in the sarcoplasm of muscle cells, where it may facilitate Ca^{2+} transport (Pauls et al., 1996).

It has been suggested that the buffering properties of parvalbumin and other calcium-binding proteins can have a neuroprotective role in the central nervous system. Parvalbumin-dependent protection against the neurotoxicity caused by calcium overload has been shown in tissue from patients with motor neuron disease or temporal lobe epilepsy (Sloviter et al., 1989; Ince et al., 1993).

Interestingly, parvalbumin and other calcium-binding proteins are specifically expressed in certain subclasses of neurons; therefore this protein is commonly used as a marker to identify specific neuronal populations. In the cerebellum parvalbumin is abundant in the Purkinje cells, and is also expressed in two subclasses of interneurons, the stellate and basket cells (Schwaller et al., 2002). The expression of parvalbumin has also been studied in the rat prefrontal cortex, where it is expressed in two subclasses of fast-spiking GABAergic interneurons,
the basket cells and the chandelier cells (Freund, 2003). Although the exact function of parvalbumin in these neurons is unclear, the Ca\(^{2+}\) buffering properties could be important for the electrophysiological characteristics of chandelier and basket neurons, influencing their capacity to exert a synchronised inhibitory firing pattern (Freund, 2003).

Functional and expression studies on parvalbumin are mainly focused on the cerebellar physiology (reviewed by Schwaller et al., 2002) and the role of this protein in the neuropathological alteration observed in the schizophrenic prefrontal cortex (Lewis et al., 2005; Lewis et al., 2004; Eyles et al., 2002). In fact, it has been reported that the schizophrenic prefrontal cortex show specific alterations of the parvalbumin-containing chandelier interneurons (Lewis et al., 2004). Parvalbumin is also likely to play a key role in the physiology of the reticular thalamus, where the mRNA encoding for this protein is very abundant, as shown by the LMD/microarray study. These interesting findings suggest that parvalbumin could be important for the neurophysiology of the GABAergic interneurons of this brain region.

4.1.4.5 Calretinin

Calretinin is another calcium-binding protein that is overexpressed in the Rt according to the LMD/microarray study. Like parvalbumin, this protein is specifically expressed in some subclasses of inhibitory interneurons; therefore the high abundance of mRNA detected in the Rt is consistent with the prevalence of GABAergic cells in this brain region.

The structure of calretinin includes six EF hands, of which five are functional and can bind Ca\(^{2+}\) ions (Schwaller et al., 1997). The polypeptide sequence forming the EF hands is very conserved in different types of calcium-binding proteins, although small differences influence the cation specificity and kinetics (Schwaller et al., 2002). Interestingly, the sequences of the non-functional EF
hands are also conserved in several calcium-binding proteins; these "degraded" structures may have an unknown functional importance.

Unlike parvalbumin, the Ca\(^{2+}\) binding sites of calretinin are highly specific, so this protein does not bind other divalent cation such as Mg\(^{2+}\) (Haiech et al., 1979). The Ca\(^{2+}\)-binding kinetic is another important biophysical property that is different in parvalbumin and calretinin; in fact the latter is considered a fast buffer, whereas the former is a slow-onset buffer (Lee et al., 2000; Schwaller et al., 2002). This property could be crucial for the specific physiological functions of the neurons where these proteins are expressed.

The exact functions of calretinin are still hypothetical, despite the abundance of data on the structure and biochemical properties of this protein. Like other calcium-binding proteins, the Ca\(^{2+}\) buffering capacity has been associated to a neuroprotective effect in neurons. Increased resistance to induced excitotoxicity has been observed \textit{in vivo} in neurons contained calretinin compared to neurons that do not express this protein (Mockel et al., 1994; Terro et al., 1998); the neuroprotective properties of calretinin have also been observed in cultured neurons (Lukas et al., 1994).

Along with the neuroprotective properties, it can be hypothesized that the Ca\(^{2+}\) buffering capacity of calretinin can be important for a number of biological processes, especially in excitable cells such as neurons, where the calcium homeostasis is crucial to their physiological functions. In fact calretinin can be part of a complex regulatory mechanism controlling the intracellular calcium concentration that also include membrane channels, intracellular compartments involved in calcium sequestration and release, and other calcium-binding proteins (Schwaller et al., 2002).
4.2 Aims

The aim of this validation study is to confirm the regional expression profile of 13 genes using ISH. These genes were selected according to their localised expression shown by the LMD/microarray study and their function.
4.3 Results

4.3.1 Genes overexpressed in the PrL

4.3.1.1 FXYD6

The autoradiograms (figure 4.1) showed that FXYD is strongly expressed in the medial prefrontal cortex, with the PrL showing a very strong signal (figure 4.1A). A qualitative analysis suggested that in this region the expression varies with the cortical layers, as the signal was stronger in layer II and in the deep layers (figure 4.1E). The intensity of the signal was very low in the white matter, in the VO and in the motor cortex, whereas the transcript was present in the medial orbital and lateral orbital cortex. The expression levels were minimal in the thalamus and the hippocampus, but high in the hypothalamus (figure 4.1B).

Quantitative analysis and comparison of FXYD6 expression levels in the PrL, VO, Rt and Ml was concordant with the expression pattern shown by the LMD/microarray study (figure 4.1). The ANOVA revealed that the expression levels in the regions examined were significantly different for FXYD6 ($F(1,5) = 212.3; p<0.001$); the simple contrasts showed that the level of expression in the PrL was significantly higher than in the VO ($F(1,5) = 190.7; p<0.001$), the Ml ($F(1,5) = 224.4; p<0.001$) and the Rt ($F(1,5) = 249.6; p<0.001$).
Figure 4.1
Figure 4.1

A. *In situ* hybridisation (ISH) autoradiogram showing the expression of FXYD6 at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Note the differential expression of FXYD6 in the cortical layers of the medial prefrontal cortex (panel E). Scale bar: 5mm.

B. ISH autoradiogram showing the expression of FXYD6 at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of FXYD6 expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(0.076,5.348)} = 212.3; p<0.001$); the simple contrasts showed that the level of expression in the PrL was significantly higher than in the VO ($F_{(0.5)} = 190.7; p<0.001$), the M1 ($F_{(0.3)} = 224.4; p<0.001$) and the Rt ($F_{(0.2)} = 249.6; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to FXYD6 showing that this gene is overexpressed in the PrL compared to VO, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).

E. ISH autoradiogram showing the differential expression of FXYD6 in the cortical layers of the medial prefrontal cortex.
4.3.1.2 GABA\(_{A}\)\(\beta 1\)

The quantitative analysis confirmed the overexpression of this gene in the PrL compared to the VO, M1 and Rt (figure 4.2). The ANOVA revealed that the regional differential expression measured was significant \((F(3,15)= 40.214; p<0.001)\); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO \((F(1,5)= 333.661; p<0.001)\), the M1 \((F(1,5)= 66.546; p<0.001)\) and the Rt \((F(1,5)= 51.248; p<0.001)\).

The ISH results fully correspond to the expression patterns shown by the LMD/microarray study, showing a slightly more pronounced overexpression of this gene in the PrL.

Concordant with the literature, the autoradiograms also showed that the expression of the GABA\(_{A}\) receptor \(\beta 1\) subunit is prominent in the hippocampus and very low or absent in the thalamic area (figure 4.2B).
Figure 4.2

A. In situ hybridisation (ISH) autoradiogram showing the expression of GABA\(_{\alpha1}\) at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Scale bar: 5 mm.

B. ISH autoradiogram showing the expression of GABA\(_{\alpha1}\) at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of GABA\(_{\alpha1}\) expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different (F(0,15) = 40.214; p < 0.001); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO (F(0,5) = 333.661; p < 0.001), the M1 (F(0,5) = 66.546; p < 0.001) and the Rt (F(0,5) = 51.248; p < 0.001).

D. Average signal intensities of microarray hybridisation for the probe set specific to GABA\(_{\alpha1}\) showing that this gene is overexpressed in the PrL compared to VO, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
Wolframin showed a very specific expression pattern. The autoradiograms (figure 4.3A) showed a strong signal in the medial prefrontal cortex, with very little or no expression in the white matter and other cortical areas. Hybridisation was very prominent in the superficial layers of the medial prefrontal cortex, with layer I showing the strongest signal (figure 4.3E). The signal intensity was decreased in layer II, and little or no expression was detected in layer III and the deep layers (figure 4.3E). The regional specificity was also evident at the level of the anterior thalamus: wolframin mRNA was abundant in the caudate-putamen, whereas it was absent in the thalamic nuclei; a very faint signal was detected in the hippocampal region (figure 4.3B).

The quantitative analysis fully reflected the expression pattern shown by the LMD/microarray study. Not only was the overexpression of this gene in the PrL confirmed, but also the small expression differences in the VO, M1 and Rt were consistently observed using both methodologies. Thus, there was a striking correspondence of the relative expression levels measured using the two independent techniques (figure 4.3C and 4.3D). The one way ANOVA showed that the regional expression was significant ($F(3,5)= 48.173; p<0.001$); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO ($F(1,5)= 87.381; p<0.001$), the M1 ($F(1,5)= 52.215; p<0.001$) and the Rt ($F(1,5)= 58.688; p<0.001$).
Figure 4.3
Figure 4.3

A. In situ hybridisation (ISH) autoradiogram showing the expression of wolframin at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Note the differential expression in the cortical layers of the medial prefrontal cortex (panel E). Scale bar: 5 mm.

B. ISH autoradiogram showing the expression of wolframin at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of wolframin expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(3,15)} = 48.173; p<0.001$); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO ($F_{(1,5)} = 87.381; p<0.001$), the M1 ($F_{(1,5)} = 52.215; p<0.001$) and the Rt ($F_{(1,5)} = 58.688; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to wolframin showing that this gene is overexpressed in the PrL compared to VO, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).

E. ISH autoradiogram showing the differential expression of FXYD6 in the cortical layers of the medial prefrontal cortex.
4.3.1.4 Synapsin IIa

The LMD/microarray study showed that the isoform synapsin IIa is overexpressed in the PrL. Although the differential expression was highly significant, with a FDR lower than 1%, the regional differential expression was not as pronounced as for FXRD6 and wolframin (see figures 4.1A, 4.3A and 4.4A).

The autoradiograms showed that synapsin IIa is expressed throughout the cortical areas at the prefrontal cortex level, although the signal was remarkably stronger in the medial prefrontal cortex (figure 4.4A). The synapsin IIa mRNA was also clearly present in the Rt (figure 4.4B). In the structures visible at the anterior thalamus level, the signal was very prominent in the hippocampal formation, paraventricular thalamic nucleus and centrolateral/paracentral thalamic nuclei (figure 4.4B).

The quantitative analysis confirmed the expression profiles shown by the LMD/microarray study (figure 4.4). The ANOVA test revealed that Synapsin IIa regional differential expression was significant ($F(3,15)=20.271$; $p<0.001$); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO ($F(1,5)= 61.912; p<0.001$), the Ml ($F(1,5)= 47.916; p<0.001$) and the Rt ($F(1,5)= 16.015; p<0.05$).
Figure 4.4

A. *In situ* hybridisation (ISH) autoradiogram showing the expression of synapsin IIa at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Scale bar: 5 mm.

B. ISH autoradiogram showing the expression of synapsin II at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of synapsin IIa expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(3,15)}$=20.271; $p<0.001$); the simple contrast showed that the level of expression in the PrL was significantly higher than in the VO ($F_{(1,5)}$= 61.912; $p<0.001$), the M1 ($F_{(1,5)}$= 47.916; $p<0.001$) and the Rt ($F_{(1,5)}$= 16.015; $p<0.05$).

D. Average signal intensities of microarray hybridisation for the probe set specific to synapsin II showing that this gene is overexpressed in the PrL compared to VO, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.2 Genes overexpressed in the VO

4.3.2.1 GABA\(_A\)\(_\delta\)

The autoradiograms showed that GABA\(_A\)\(_\delta\) is expressed in the prefrontal cortex, with a stronger signal in the superficial layers (figure 4.5A).

The quantitative analysis confirmed the expression profiles shown by the LMD/microarray study (figure 4.5C and 4.5D). The ANOVA test revealed that GABA\(_A\)\(_\delta\) regional differential expression was significant (\(F(3,12)=31.921; p<0.001\)); the simple contrast showed that the level of expression in the VO was significantly higher than in the PrL (\(F(1,4)=159.880; p<0.001\)), the M1 (\(F(1,4)=17.766; p<0.05\)) and the Rt (\(F(1,4)=23.980; p<0.05\)).

The autoradiograms also showed that this gene is expressed in the thalamus, in the striatum and in the dentate gyrus of the hippocampus (figure 4.5B). These observations are concordant with the distribution profiles reported in the literature (Pirker et al., 2000; Wisden et al., 1992).
Figure 4.5

A. In situ hybridisation (ISH) autoradiogram showing the expression of GABA_{A,\delta} at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Scale bar: 5 mm.

B. ISH autoradiogram showing the expression of GABA_{A,\delta} at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of GABA_{A,\delta} expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(3,12)} = 31.921; p<0.001$); the simple contrast showed that the level of expression in the VO was significantly higher than in the PrL ($F_{(1,4)} = 159.880; p<0.001$), the M1 ($F_{(1,4)} = 17.766; p<0.05$) and the Rt ($F_{(1,4)} = 23.980; p<0.05$).

D. Average signal intensities of microarray hybridisation for the probe set specific to GABA_{A,\delta} showing that this gene is overexpressed in the VO compared to PrL, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.2.2 CaMKII inhibitor α

The LMD/microarray study predicted that CaM-KIIα is overexpressed in the VO; however the differential expression between this area and the M1 was significant but very small (figure 4.6A and 4.6C). In fact, the VO overexpressed CaM-KIIα compared to the M1 with a statistical significance of 0.93% FDR, very close to the 1% threshold chosen for the LMD/microarray study and a Fold Change of 1.51 (see table 3.4, “Phosphorylation” functional class). The confirmation of this result using an independent alternative technique such as ISH was useful from this technical point of view.

The autoradiograms showed that CaM-KIIα is expressed in the cortical regions visible at the prefrontal cortex level (figure 4.6A). Concordant with published studies (Chang et al., 2001), the signal was more prominent in the layer II and III of the cortex compared to the deep layers.

The quantitative analysis confirmed the expression patterns shown by the LMD/microarray study (figure 4.6c and 4.6D). The ANOVA revealed that the regional differential expression of CaM-KIIα was significant ($F_{(3,15)}=113.6; p<0.001$); the simple contrast showed that the level of expression in the VO was significantly higher than in the PrL ($F_{(1,5)}=73.6; p<0.001$), the M1 ($F_{(1,5)}=6.9; p<0.05$) and the Rt ($F_{(1,5)}=296.2; p<0.001$). Interestingly, the ISH analysis confirmed that the small overexpression of CaM-KIIα in the VO compared to the M1 was significant.

Additionally, the signal detected in the caudate-putamen was also concordant with the results reported by Chang et al (2001); however, this group reported a prominent expression of CaM-KIIα in the hippocampal formation that could not be observed in the present experiments.
Figure 4.6

A. In situ hybridisation (ISH) autoradiogram showing the expression of CaM-KIIα at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows. Scale bar: 5mm.

B. ISH autoradiogram showing the expression of CaM-KIIα at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow.

C. Quantification of CaM-KIIα expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F(3,15)= 113.6; p<0.001$); the simple contrast showed that the level of expression in the VO was significantly higher than in the PrL ($F(1,5)= 73.6; p<0.001$), the M1 ($F(1,5)= 6.9; p<0.05$) and the Rt ($F(1,5)= 296.2; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to CaM-KIIα showing that this gene is overexpressed in the VO compared to PrL, M1 and Rt. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.3 Genes overexpressed in the Rt

4.3.3.1 RGS3

RGS3 was selected for validation because the overexpression the Rt detected by the microarray study has a great biological significance for neurotransmission and modulation of signal transduction. Additionally, the expression levels detected in the Rt by the LMD/microarray analysis showed an unusually high variability across the biological replicates (figure 4.7D). However, this variability did not affect the statistical significance of the overexpression, as the False Discovery Rate associated with this gene was ≤ 1%.

The autoradiograms showed that RGS3 is not expressed in the prefrontal cortex (figure 4.7A). At the anterior thalamus level, the mRNA encoding for RGS3 was detected in the thalamus. Interestingly, the Rt showed expression only in the ventral part, whereas the dorsal Rt showed a very faint signal (figure 4.7B).

The quantitative analysis was performed measuring the expression levels in the dorsal and ventral Rt (figure 4.7C); the ANOVA test revealed that the differential regional expression was significant ($F_{(2,5)}= 169.176; p<0.001$). The simple contrast showed that the level of expression in the ventral Rt was significantly greater than in the dorsal Rt ($F_{(1,5)}= 134.678; p<0.001$), the PrL ($F_{(1,5)}= 265.222; p<0.001$), the VO ($F_{(1,5)}= 242.244; p<0.001$), and the M1 ($F_{(1,5)}= 308.699; p<0.001$).
Figure 4.7
A. *In situ* hybridisation (ISH) autoradiogram showing that RGS3 is not expressed at the level of prefrontal cortex.

B. ISH autoradiogram showing the expression of RGS3 at the level of anterior thalamus. The anatomical localisation of the ventral and dorsal reticular thalamic nucleus (Rt) is indicated by arrows. Scale bar: 5 mm.

C. Quantification of RGS3 expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(1.854,9.271)} = 169.176; p<0.001$). The simple contrast showed that the level of expression in the ventral Rt was significantly greater than in the dorsal Rt ($F_{(1.5)} = 134.678; p<0.001$), the PrL ($F_{(1.5)} = 265.222; p<0.001$), the VO ($F_{(1.5)} = 242.244; p<0.001$); and the M1 ($F_{(1.5)} = 308.699; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to RGS3 showing that this gene is overexpressed in the Rt compared to PrL, VO and M1. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).

153
4.3.3.2 PLCβ4

The autoradiograms showed very low signal at the level of the prefrontal cortex, although some transcript was detected in the superficial layers of the motor cortex (figure 4.8A). At the anterior thalamus level, the expression of PLCβ4 was prominent in the thalamic nuclei, including the Rt, whereas the signal was very low or absent in the other brain structures visible at this level (figure 4.8B). Consistent with the expression pattern observed at the prefrontal cortex level, a low degree of expression was detectable in the superficial layers of the cortex.

The quantitative analysis of PLCβ4 expression in the regions of interest confirmed the expression pattern shown by the LMD/microarray study (figure 4.8C and 4.8D). The ANOVA test revealed that the differential regional expression was significant ($F_{(3,5)} = 128.836; p<0.001$). The simple contrast showed that the level of expression in the Rt was significantly higher than in the PrL ($F_{(1,5)} = 961.633; p<0.001$), the VO ($F_{(1,5)} = 320.408; p<0.001$) and the M1 ($F_{(1,5)} = 131.723; p<0.001$).
Figure 4.8

A. *In situ* hybridisation (ISH) autoradiogram showing the expression of PLCβ4 at the level of prefrontal cortex.

B. ISH autoradiogram showing the expression of PLCβ4 at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of PLCβ4 expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F(3,15) = 128.836; p<0.001$); The simple contrast showed that the level of expression in the Rt was significantly higher than in the PrL ($F(1,5) = 961.633; p<0.001$), the VO ($F(1,5) = 320.408; p<0.001$) and the M1 ($F(1,5) = 131.723; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to PLCβ4 showing that this gene is overexpressed in the Rt compared to PrL, VO and M1. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.3.3 PKCô

Similar to RGS3 (see section 4.3.3.1), the LMD/microarray detected a highly significant overexpression of PKCô (FDR ≤ 1%) in the Rt compared to the cortical regions examined; however, the average level of expression of this gene in the Rt showed an unusually high standard error (figure 4.9D). For this reason and for its biological importance, PKCô was selected for validation by ISH.

The autoradiograms showed that PKCô is not expressed in any structure at the level of the prefrontal cortex (figure 4.9A). The mRNA was detected in the thalamus, while there was no signal in other structures such as the hypothalamus and the hippocampus.

Interestingly, the signal was only present in the ventral part of the Rt, whereas the dorsal Rt did not show expression of PKCô (figure 4.9B). This expression profile was very similar to that of RGS3 (see section 4.3.3.1).

The quantitative analysis was performed measuring the expression levels in the dorsal and ventral Rt (figure 4.9C); The ANOVA test revealed that the differential regional expression was significant ($F(1,3426.709) = 173.710; p<0.001$); The simple contrast showed that the level of expression in the ventral Rt was significantly different to the dorsal Rt ($F(1,5) = 217.244; p<0.001$), the PrL ($F(1,5) = 197.514; p<0.001$), the VO ($F(1,5) = 234.914; p<0.001$); and the M1 ($F(1,5) = 169.019; p<0.001$).
Figure 4.9

A. In situ hybridisation (ISH) autoradiogram showing that PKCδ is not expressed at the level of prefrontal cortex.

B. ISH autoradiogram showing the expression of PKCδ at the level of anterior thalamus. The anatomical localisation of the ventral and dorsal reticular thalamic nucleus (Rt) is indicated by arrows. Scale bar: 5 mm.

C. Quantification of PKCδ expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F_{(1,342,6.709)} = 173.710; p<0.001$); The simple contrast showed that the level of expression in the ventral Rt was significantly different to the dorsal Rt ($F_{(1,5)} = 217.244; p<0.001$), the PrL ($F_{(1,5)} = 197.514; p<0.001$), the VO ($F_{(1,5)} = 234.914; p<0.001$); and the M1 ($F_{(1,5)} = 169.019; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to PKCδ showing that this gene is overexpressed in the Rt compared to PrL,VO and M1. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.3.4 Parvalbumin

Concordant with the literature, the autoradiograms showed that parvalbumin is expressed in all cortical areas at the prefrontal cortex level (figure 4.10A). At the level of the anterior thalamus, the parvalbumin-specific radioactive probe produced a very intense signal corresponding to the Rt (figure 4.10B).

The quantitative analysis of parvalbumin expression in the regions of interest confirmed the expression pattern shown by the LMD/microarray study (figure 4.10C and 4.10D). The ANOVA revealed that the differential regional expression was significant ($F(1,5) = 1480.958; p<0.001$); the simple contrasts showed that the level of expression in the Rt was significantly higher than in the PrL ($F(1,5) = 2152.971; p<0.001$), the VO ($F(1,5) = 1239.496; p<0.001$) and the M1 ($F(1,5) = 1675.988; p<0.001$).

Additionally, parvalbumin expression was also observed in the hippocampus and the lateral globus pallidus.
Figure 4.10

A. In situ hybridisation (ISH) autoradiogram showing the expression of parvalbumin at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows.

B. ISH autoradiogram showing the expression of parvalbumin at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of parvalbumin expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F(1.204,6.016)=1480.958; p<0.001$); the simple contrasts showed that the level of expression in the Rt was significantly higher than in the PrL ($F(1.5)=2152.971; p<0.001$), the VO ($F(1.5)=1239.496; p<0.001$) and the M1 ($F(1.5)=1675.988; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to parvalbumin showing that this gene is overexpressed in the Rt compared to PrL, VO and M1. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.3.3.5 Calretinin

The autoradiograms showed that the expression levels of calretinin are very low at the level of the prefrontal cortex, whereas the signal was very prominent in the Rt (figure 4.11A and 4.11B).

The quantification of the relative optical density in the regions of interest confirmed the expression profile shown by the LMD/microarray study (figure 4.11C and 4.11D). The ANOVA test showed that the regional differential expression of calretinin is significant ($F(1,5) = 80.008; p<0.001$); the simple contrast showed that the level of expression in the Rt was significantly greater than in the PrL ($F(1,5) = 80.082; p<0.001$), the VO ($F(1,5) = 80.900; p<0.001$) and the M1 ($F(1,5) = 79.674; p<0.001$).

Additionally, the calretinin specific radioactive probe showed strong signal in other thalamic nuclei, such as the paraventricular nucleus, the ventromedial nucleus and the reuniensis and rhomboid nuclei. The hypothalamus also showed a uniform signal showing that calretinin is expressed in this area.
Figure 4.11

A. In situ hybridisation (ISH) autoradiogram showing the expression of calretinin at the level of prefrontal cortex. The anatomical localisation of the prelimbic cortex (PrL), ventral orbital cortex (VO), primary motor cortex (M1) is indicated by arrows.

B. ISH autoradiogram showing the expression of calretinin at the level of anterior thalamus. The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of calretinin expression in the PrL, VO, M1 and Rt by optical densitometry of ISH autoradiograms. Concordant with the microarray results (D), the expression levels in the regions examined were significantly different ($F(1,0.975,0.035) = 80.008; p<0.001$); the simple contrast showed that the level of expression in the Rt was significantly greater than in the PrL ($F(1,5) = 80.082; p<0.001$), the VO ($F(1,5) = 80.900; p<0.001$) and the M1 ($F(1,5) = 79.674; p<0.001$).

D. Average signal intensities of microarray hybridisation for the probe set specific to calretinin showing that this gene is overexpressed in the Rt compared to PrL, VO and M1. Statistical significance is expressed by False Discovery Rate (FDR ≤ 1%).
4.4 Discussion

4.4.1 Regional expression analysis by LMD-microarray and ISH: technical considerations

In this study *in situ* hybridisation was successfully employed for the validation of regional gene expression profiles detected using the LMD-microarray strategy. In fact, ISH allowed reproduction of the microarray results in a completely independent manner; moreover it produced additional information on the regional expression and clarified the laminar expression in the cortical areas of selected genes.

Comparison of the results obtained with the two techniques showed that the LMD/microarray approach is more sensitive than ISH in detecting low amounts of RNA in specific brain regions. The greater sensitivity is essential to detect differences of low abundance transcripts. The genes Doublecortin (Dcx) and Serine/threonine kinase 23 (Stk23) could not be detected by ISH, whereas the LMD-microarray showed regional overexpression in the PrL and in the VO respectively (see table 3.3, “Neurogenesis/neurodevelopment” functional class and table 3.4, “Phosphorylation” functional class). The linear amplification could contribute to the increased sensitivity of the LMD-microarray approach. Considering that the differential expression of the detectable genes measured by ISH always validated the LMD-microarray results, these findings suggest that the linear amplification not only preserves reliably the relative abundance of the mRNAs, but also increases the sensitivity allowing the detection of transcripts that would appear unexpressed according to a conventional technique such as ISH.

Although a full verification of the linearity of the amplification procedure was beyond the scope of this study, as the reliability of this technique has been
previously tested (Schneider et al., 2004; Patel et al., 2005), ISH results showed that the relative abundance of the mRNA of the genes investigated had not been altered in the amplification step for the genes selected for validation.

These findings confirm the vast potential of the LMD-microarray approach in neuroscience demonstrating that this technique is applicable to the study of differential gene expression in rat cortical subregions. Furthermore, the ISH results provided additional information about the intra-regional differential expression of PKCδ and RGS3 (see below); hence this technique not only was useful to confirm the validity of the LMD/microarray procedure, but also complemented the study with original observations.

4.4.2 Validation of genes overexpressed in the PrL

The ISH study confirmed the overexpression of four genes in the PrL detected using the LMD/microarray strategy. In all cases the expression profiles measured using the two techniques were remarkably similar both qualitatively and quantitatively.

FXYD6 showed the most specific expression pattern, as this transcript is found exclusively in the medial prefrontal cortex, and in particular in the PrL (figure 4.1). The biological significance of this interesting and original finding is likely to be relevant to the ion trafficking and neurotransmission in the PrL, as this gene has a crucial role in the modulation of the Na-K-ATPase (see section 4.1.2.1). Although previous reports suggest that the members of FXYD family are tissue specific (see above), this is the first time that specific expression of FXYD6 is demonstrated at the level of subcortical region using two independent techniques. This finding suggests that the activity of the Na-K-ATPase, which is crucial for the function of excitable cells, is differentially regulated at a very specific anatomical level in the rat brain.
ISH results also provided interesting information about the expression of Wolframin in the PrL. The regional expression pattern of this gene was confirmed and the quantitative measurements using the two techniques were strikingly similar (figure 4.3). Moreover, the ISH autoradiograms highlighted that Wolframin is differentially expressed in the cortical layers (figure 4.3E). In fact, the autoradiogram showed a very strong signal in the superficial layers of the PrL. The intensity of the signal decreased gradually in the layer II; while the transcript appeared absent in the deep cortical layers. This expression pattern could not be detected by the LMD/microarray study, as the whole PrL was dissected. This finding shows that ISH not only is useful for the validation of LMD/microarray results, but also provides original information that may be very relevant for the neurobiology of the region examined.

The confirmation of the overexpression of GABA\textsubscript{A}β1 and synapsin II in the PrL was important for the function of these genes. GABA\textsubscript{A}β1 was classified as a gene directly involved in neurotransmission after the Gene Ontology analysis of the LMD/microarray results. Synapsin II is also involved in the control of neurotransmission, as this protein controls synaptic function and modulates exocytosis of synaptic vesicles (see section 4.1.2.3). A number of genes belonging to this functional group showed regional overexpression according to the LMD/microarray study (see table 3.3). Although it would be unpractical to confirm the differential regional expression of all the neurotransmission-related genes, the validation of GABA\textsubscript{A}β1 and synapsin II strongly supports the finding that the genes belonging to this functional class have a specific pattern of expression in the regions examined.

The overexpression of doublecortin (Dcx) in the PrL could not be confirmed by ISH, as the expression levels of this gene was below the sensitivity limit of this technique. This finding suggest that the amplification step included in the LMD/microarray strategy contributes to an improved sensitivity compared to techniques that detect the mRNA originally present in the sample, such as ISH. The increased sensitivity can be an important feature, as the differences observed using the LMD/microarray approach are likely to be biologically relevant. This
finding also suggests that the validation of genes with low basal levels of expression might be better achieved using amplification-based techniques, such as real-time RT-qPCR.

4.4.3 Validation of genes overexpressed in the VO

The validation of the overexpression of CaM-KIINα in the VO demonstrated that the LMD/microarray strategy allows the detection of small expression differences with a high degree of reliability. In fact, the differential expression of this gene between the VO and M1 was associated with a False Discovery Rate of 0.93%, which is very close to the 1% threshold chosen for this study. The quantitative difference was also marginal, with a Fold Change of 1.51.

The correspondence between the data obtained from the LMD/microarray study and the ISH validation experiment was remarkable both qualitatively and quantitatively (figure 4.6). In this case, the differential expression measured by ISH between the VO and the M1 was slightly smaller than that measured using the microarray strategy (figure 4.6). Similarly to the LMD/microarray, the difference measured by ISH was associated with a p value of 0.047, which is very close to the 0.05 threshold chosen for statistical significance. This finding suggests that the LMD/microarray approach can detect small expression differences as reliably as ISH, despite the complex manipulations of the RNA involved in this procedure, such as the linear amplification.

The validation of GABAAδ was also remarkable, as this result confirm that the expression of GABAA receptor subunits is highly localised in specific cortical subregions. Similar to CaM-KIINα, the differential expression of GABAAδ between the VO and the M1 was associated with a relatively low Fold Change of 1.54. Even in this case, the ISH could validate the small difference detected using
the LMD/microarray strategy, confirming again the validity and the reliability of this approach.

Validation of the overexpression of the gene encoding for serine-threonine kinase 23 (Stk23) in the VO was also attempted. Similar to Dcx (see above), this transcript was below the limit of detection of ISH, confirming that the increased sensitivity of the LMD/microarray approach is advantageous to detect differences when the gene of interest has a low level of expression.

4.4.4 Validation of genes overexpressed in the Rt

The overexpression of five genes in the Rt was validated by ISH (figures 4.7 to 4.11).

The LMD/microarray study showed a strong overexpression of the calcium binding proteins parvalbumin and calretinin in the Rt. The ISH experiment validated this finding, confirming that high level of expression of these genes is a landmark of the Rt. Although both parvalbumin and calretinin are expressed in the cortical regions examined (figures 4.10 and 4.11), their overexpression in the Rt is due to the neuronal composition of this nucleus. In fact the Rt is mainly composed of GABAergic interneurons that are known to express calcium-binding proteins (Paxinos, 1995).

PLCβ4 is another gene that was overexpressed in the Rt according to the LMD/microarray study and the ISH confirmation experiments. PLCβ4 plays a crucial role in the transduction of the signal initiated by the activation of receptors coupled with Gq proteins (see above), and is likely to be relevant to the neurobiology of the Rt. Moreover, other genes involved in the same biological pathway such as RGS3 and PKCδ were also overexpressed in the Rt (see above); therefore the validation of the expression pattern of PLCβ4 predicted by the LMD/microarray study is useful to confirm these interesting results.
The LMD/microarray study also predicted the overexpression of PKCβ and RGS3 in the Rt. These genes are directly involved in the neurotransmission mediated by G-protein coupled receptors. The ISH confirmation of their regional expression pattern strongly supported the finding that a number of genes belonging to this functional group are overexpressed in the Rt. Interestingly, the data obtained from the ISH experiment not only confirmed the observations from the LMD/microarray study, but also provided original information about the sub-regional expression pattern of PKCβ and RGS3 within the Rt (figures 4.7 and 4.9). The autoradiograms showed that these two genes are overexpressed in the ventral part of the Rt only, whereas the expression is marginal in the dorsal Rt. This finding confirms the biological importance of localised gene expression in brain regions and also suggests that the expression of some genes can be regulated at a sub-regional level in the Rt.
CHAPTER 5. EFFECT OF CHRONIC PCP TREATMENT ON EXPRESSION OF GENES ENRICHED IN THE PR, VO AND RT
5.1 Introduction

Phencyclidine (PCP) (figure 5.1) is a psychotomimetic compound that has been popular as an illicit recreational drug for its hallucinatory effects. Although it was first developed as a surgical anaesthetic, the therapeutic utility of PCP was severely limited by prominent side effects such as hallucinations, disordered speech, delirium, agitation and disoriented behaviour. Interestingly, these effects resemble very closely the symptoms experienced by patients suffering from schizophrenia; therefore PCP has been extensively used in neuroscience research to model aspects of this disease (Morris et al., 2005).

Compounds with similar pharmacology, such as MK-801 and ketamine, are also useful research tools for their psychotomimetic properties (figure 5.1) (Tamminga et al., 2003). Ketamine, which is similar to PCP both for the chemical structure and the pharmacology, has similar effects on the central nervous system. The psychotomimetic properties of this drug are less pronounced than PCP; therefore it is still used in veterinary and paediatric anaesthesia.

![Figure 5.1](image)

Chemical structures of the NMDA channel blockers PCP, Ketamine and MK-801.
5.1.1 Pharmacology of PCP

PCP binds a number of different receptors in the central nervous system; hence the pharmacology of this drug is rather complex (Morris et al., 2005).

PCP is mainly a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) glutamate receptor. This drug is classified as an open channel blocker, as its site of binding is within the channel pore and it is accessible to the compound only when the channel is open (Ferrer-Montiel et al., 1998). The NMDA receptors differ from the other types of ionotropic glutamate receptor for their specific permeability to $\text{Ca}^{2+}$, for the voltage dependent blockade exerted by $\text{Mg}^{2+}$ and for the presence of the specific N/Q/R residue group within the channel that forms the binding site for the open channel blockers such as PCP (Mayer et al., 1994; Mayer et al., 1987; Zarei et al., 1994; Ferrer-Montiel et al., 1998). The NMDA receptors are heteroligomers composed of NR1 subunits and one or more NR2 subunits (NR2A-D) that are probably arranged in a tetrameric structure (Hollmann et al., 1994; Ferrer-Montiel et al., 1996). The pore-lining structure is composed by the M2 transmembrane segment present in each subunit; this domain determines the ion specificity of the NMDA receptor and contains the residues forming the binding site for PCP (Sakurada et al., 1993; Ferrer-Montiel et al., 1995).

PCP also inhibits other ion channels, such as voltage-gated sodium and potassium channels, by binding to a specific site within the pore (FFrench-Mullen et al., 1989). The nicotinic acetylcholine receptor is another ion channel inhibited by PCP in a non-competitive manner (Oswald et al., 1984). Moreover, it has been shown that this drug also interacts with sigma receptors and the dopamine and noradrenaline transporters (Garey et al., 1976; Contreras et al., 1988; Pubill et al., 1998).

The interaction of PCP with the NMDA receptors is by far the most potent and it is likely to be the most biologically relevant mechanism of action of PCP-induced effects in the central nervous system at the doses commonly used.
However the interaction of this drug with other ion channels and neurotransmitters receptors may contribute to its unique pharmacological profile and could play a role in the complex psychotomimetic properties of PCP (Morris et al., 2005).

5.1.2 Psychotomimetic effects of PCP in humans

The PCP-induced psychosis observed after administration of this drug to normal humans was first reported by Luby et al (1959). This group also found that PCP exacerbates symptoms in schizophrenic patients. Studies of the effects of PCP in humans were soon abandoned due to ethical reasons.

Similar, but less pronounced psychotomimetic effects were described for ketamine, a congener of PCP (Rainey et al., 1974). In fact, competitive NMDA antagonist also can reproduce some positive symptoms of schizophrenia in healthy subjects, suggesting that the interference with the glutamatergic transmission is a key feature of psychotomimetic drugs (Tamminga et al., 1998). However, PCP is unique in reproducing not only the positive symptoms of schizophrenia, such as hallucinations, delusions and thought disorder, but also some of the negative symptoms such as social withdrawal. Moreover, there is evidence that the NMDA open channel blockers, especially PCP, can also cause some forms of severe cognitive impairment. It has been reported that after administration of PCP, subjects showed deficits in memory, verbal fluency and impaired performance in the Wisconsin Card Sort Test. These findings show that PCP is able to reproduce not only the active psychotic symptoms of schizophrenia, but also the persistent cognitive dysfunctions that are considered a landmark of this disease (Krystal et al., 1994; Jentsch et al., 1999).

The popularity of PCP as an illicit recreational drug has allowed the collection of data on the long-term effects of this drug in humans (reviewed by
Jentsch et al., 1999). Interestingly, the chronic administration of PCP not only prolongs the duration of some positive psychotomimetic effects, but also induces some qualitatively different alterations compared to the single-dose administration. For example, the PCP-induced hallucinations tend to be visual after a single administration of the drug; however, subjects report prolonged auditory and paranoid hallucinatory episodes after chronic exposure (Allen et al., 1978; Kristal et al., 1994). These interesting findings suggest that the psychosis induced by prolonged exposure to PCP resembles the schizophrenia positive symptoms more closely than the acute effects of the drug (Jentsch et al., 1999). Another striking qualitative difference between the chronic and the acute effect of NMDA open channel blockers regards the frontal blood flow. This parameter is transiently increased after acute exposure to ketamine; however chronic administration of PCP causes a persistent decrease of the blood flow in the frontal lobe and reduced glucose utilisation (Breier et al., 1997; Hertzman et al., 1990). These long-term effects could be related to the specific cognitive impairments induced by chronic PCP and closely mirror hypofrontality, which is the reduced blood flow and metabolism in the frontal brain region observed in the schizophrenic brain.

In summary, the available data about the effects of PCP in humans suggest that the psychosis induced by this drug closely mirrors different aspects of the schizophrenia symptomatology, especially after prolonged exposure (Jentsch et al., 1999).

5.1.3 The utility of PCP in modeling aspects of schizophrenia in animals

Modeling aspects of schizophrenia in animals is very useful for neuroscience research. Therefore, many studies investigate the effects of PCP in non-primate mammals, in order to establish whether this drug could induce neurochemical and
behavioural alterations that mirror some of the abnormalities observed in the human diseased brain.

The consequences of acute administration of PCP to rodents include an increased expression of immediate early genes in the prefrontal cortex, indicating increased neuronal activity in this brain region (Gao et al., 1998). This initial stimulation is followed by a delayed decrease of the prefrontal cortical neuronal activity (Gao et al., 1993; Gao et al., 1998). Similar results were obtained using MK-801, another NMDA open channel blocker (Gao et al., 1998). These findings are in accordance with the increased frontal blood flow measured in humans after acute exposure to PCP, and the hypofrontality-like reduction of neuronal metabolic activity observed in the same brain region after prolonged exposure to the drug (see above). This scenario suggests that the delayed or chronic effects of PCP in rats are more likely to mirror the metabolic alterations of the schizophrenic brain.

When evaluating the schizophrenia-like alterations induced by PCP in animals, it is important to take into account the neurotoxicity of this drug. It has been demonstrated that a very high dose (50 mg/kg) of PCP can cause irreversible cell death in the rat brain (Olney et al., 1995; Corso et al., 1997); repeated administration of moderately high doses (15 mg/kg) also causes neuronal damage in the limbic brain regions (Olney et al., 1989; Corso et al., 1997). As there is no evidence of neurodegeneration in the schizophrenic brain, it can be argued that neurotoxic doses of PCP are of little use for modelling this disease.

In fact, it has been demonstrated that very low doses of PCP (2.58 mg/kg) are sufficient to reproduce striking neurochemical and behavioural schizophreniform alterations in the rat (reviewed by Morris et al., 2005). After a single low dose of PCP, rats showed impaired performance in an attentional set-shifting task that correspond to the cognitive deficits assessed by the Wisconsin Card Sort Test in schizophrenic patients (Egerton et al., 2005). Moreover, the same dose caused a decreased expression of parvalbumin in the rat prefrontal cortex, suggesting that low doses of PCP can interfere with the GABAergic neurotransmission.
reproducing the abnormalities observed in the interneurons of the schizophrenic prefrontal cortex (Cochran et al., 2002; Lewis et al., 2004).

Interestingly, the decreased expression of parvalbumin in the prefrontal cortex, which is a strong evidence for specific dysfunction of the chandelier and basket subtypes of GABAergic interneurons, was confirmed after chronic exposure to low doses of PCP (Cochran et al., 2003). Cochran et al (2003) also assessed the metabolic alterations induced by chronic low doses of PCP in multiple brain regions, showing that the glucose utilisation was decreased in the prefrontal cortex, in accordance with the hypofrontality observed in the schizophrenic brain. Metabolic alterations were also specifically observed in some auditory structures, in the hippocampus and in the reticular thalamic nucleus, whereas the majority of brain regions did not show significant changes.

The literature on PCP models of schizophrenia shows that this drug is a very useful tool to reproduce different characteristics of this psychiatric disease in rodents. The unique pharmacological profile of PCP allows modelling not only some crucial cognitive and behavioural abnormalities (Egerton et al., 2005), but also a complex range of neurochemical and metabolic alterations that are landmarks of the human disease. In fact, the hypofrontality-like decrease of metabolic activity in the prefrontal cortex and the specific alterations of parvalbumin-containing GABAergic interneurons after chronic administration of PCP (Cochran et al., 2003) strongly support the utility of this model.
5.2 Aims

In this section a well-established rat model of schizophrenia based on chronic-intermittent administration of PCP (Cochran et al. 2003) will be used to investigate possible expression changes of candidate genes that showed regional overexpression in the PrL, VO and Rt (see chapters 3 and 4). The candidate genes, selected on the basis of their regional expression patterns and their biological function, showed localised overexpression in one of the schizophrenia-susceptible region both according to the LMD/microarray study and the ISH validation study (see chapters 3 and 4).
5.3 Results

5.3.1 Genes overexpressed in the Rt

The expression of RGS3, PKC\(\delta\) and PLC\(\beta4\) was analysed in the PCP model of schizophrenia. These genes play a crucial role in the neurotransmission mediated by G protein-coupled receptor and showed a distinct overexpression in the Rt according to the LMD/microarray study and the ISH validation study (see chapters 3 and 4).

The quantitative analysis of the expression levels of RGS3, PKC\(\delta\) and PLC\(\beta4\) in the Rt of PCP-treated and control animals showed that there was no significant drug-induced expression change of these genes (figures 5.2, 5.3 and 5.4) (RGS3: \(t(10)= -0.557;\) p>0.05. PKC\(\delta\): \(t(10)= 0.267;\) p>0.05. PLC\(\beta4\): \(t(10)= 0.088;\) p>0.05).

The expression of parvalbumin and calretinin in the PCP model of schizophrenia was not investigated, as it has been described by Cochran \textit{et al} (2003).
Figure 5.2

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of RGS3 at the level of anterior thalamus, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of RGS3 expression in the ventral Rt following chronic PCP treatment by optical densitometry of ISH autoradiograms. Expression levels were measured in the ventral Rt only, as RGS3 is specifically expressed in this part of the Rt (see section 4.3.3.1). No significant difference was measured between the PCP and vehicle treated animals (t(10)= -0.557; p>0.05).
Figure 5.3

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of PLCβ4 at the level of anterior thalamus, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of PLCβ4 expression in the Rt following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(10) = 0.088; p>0.05).
Figure 5.4

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of PKCδ at the level of anterior thalamus, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of PKCδ expression in the ventral Rt following chronic PCP treatment by optical densitometry of ISH autoradiograms. Expression levels were measured in the ventral Rt only, as PKCδ is specifically expressed in this part of the Rt (see section 4.3.3.3). No significant difference was measured between the PCP and vehicle treated animals (t(10)= 0.267; p>0.05).
5.3.2 Genes overexpressed in the VO

The quantitative analysis of the expression levels of CaMKII inhibitor α in the VO of PCP-treated and control animals showed that there was no significant expression change of this gene after chronic PCP treatment (figure 5.5) (t(9)= 0.014; p>0.05). The expression of this gene after chronic PCP treatment was also analysed in the PrL, as the earlier autoradiograms showed that CaMKII inhibitor α is also expressed in this important area, although to a lesser degree than in the VO (figure 4.5). Similarly to the VO, the quantitative analysis did not show PCP-induced expression changes of CaMKII inhibitor α in the PrL (t(9)= 0.120; p>0.05).

The quantitative analysis showed that the expression of GABA_Aδ have a tendency to decrease in the VO after chronic PCP treatment. However, the statistical analysis showed that the difference measured is not significant (figure 5.6) (t(8)= 1.841; p>0.05).
Figure 5.5

A and B. *In situ* hybridisation (ISH) autoradiograms showing the expression of CaMKII inhibitor α at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the ventral orbital cortex (VO) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of CaMKII inhibitor α expression in the VO following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(9)= 0.014; p>0.05).
Figure 5.6

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of GABA_A,δ at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the ventral orbital cortex (VO) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of GABA_A,δ expression in the VO following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(8)= 1.841; p>0.05).
5.3.3 Genes overexpressed in the PrL

The expression of the four genes that were overexpressed in the PrL according to the LMD/microarray study and the ISH validation study (see chapters 3 and 4) was analysed in the PCP model of schizophrenia.

The quantitative analysis of the expression levels of FXYD6 in the PrL of PCP-treated and control animals showed that there is no significant expression change of this gene after chronic PCP treatment (figure 5.7) ($t(10)= 0.317; p>0.05$). As the autoradiograms showed that the expression of FXYD6 vary across the cortical layers of the PrL (see chapter 4) (figure 4.1), an alternative quantitative analysis was performed to investigate whether the PCP treatment caused a layer-specific expression change of this gene. This analysis did not show significant PCP-induced expression changes in layer II ($t(10)= -0.42; p>0.05$) or in the deep layers of the PrL ($t(10)= 0.653; p>0.05$).

Similarly, the expression of GABA_Aβ1 did not show significant PCP-induced changes in the PrL ($t(10)= 0.086; p>0.05$) (figure 5.8). Although this gene was clearly overexpressed in the PrL (see chapters 3 and 4), the autoradiograms showed that it is also expressed in the VO and in the hippocampus (figure 4.2); therefore the quantitative analysis was also performed in these regions, as they are relevant to crucial functions such as attention and memory. No significant expression changes were detected in the VO ($t(10)= 0.516; p>0.05$) or in the hippocampal regions CA3 ($t(9)= -1.282; p>0.05$) and dentate gyrus (DG) ($t(9)= -1.541; p>0.05$).

The quantitative analysis of the expression levels of synapsin II in the PrL of PCP-treated and control animals showed that there is no drug-induced expression change ($t(10)= 0.148; p>0.05$) (figure 5.9). The autoradiograms showed that this gene is also expressed in other regions of interest for cognition and schizophrenia, although to a lesser degree than in the PrL (refer to section 4.3.1.4). Hence, the quantitative analysis of synapsin II expression after chronic PCP treatment was also performed in the VO, in the Rt, and in the hippocampal regions CA3 and DG.
No significant expression changes were observed in the above regions (VO: $t(10) = -0.102; p>0.05$. Rt: $t(10) = -0.141; p>0.05$. CA3: $t(7) = 0.807; p>0.05$. DG: $t(7) = 1.747; p>0.05$).

Finally, the expression of Wolframin was quantified and analysed in the PrL, where this gene is overexpressed according to the LMD/microarray study and the ISH validation study (see chapters 3 and 4). Interestingly, Wolframin mRNA expression showed a significant decrease of 19.8% after chronic PCP treatment ($t(10) = 2.245; p<0.05$) (figure 5.10). Thus this is the only transcript of the 9 studied to show PCP-induced expression changes.
Figure 5.7

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of FXYD6 at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of FXYD6 expression in the PrL following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(10) = 0.317; p>0.05).
Figure 5.8

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of GABA$_{A}$$\beta$$1$ at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of GABA$_{A}$$\beta$$1$ expression in the PrL after chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals ($t(10)=0.086; p>0.05$).
Figure 5.9

*A and B.* In situ hybridisation (ISH) autoradiograms showing the expression of synapsin II at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

*C.* Quantification of synapsin II expression in the PrL following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals ($t(10)= 0.148; p>0.05$).
Figure 5.10
Figure 5.10

A and B. In situ hybridisation (ISH) autoradiograms showing the expression of wolframin at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

C and D. Pseudocolour autoradiograms corresponding to A and B. Correspondence between colour and relative optical density (ROD) is shown on the left side of each autoradiogram. The anatomical localisation of the PrL is indicated by an arrow.

D. Quantification of wolframin expression in the PrL following chronic PCP treatment by optical densitometry of ISH autoradiograms. This gene was significantly downregulated in the PCP treated animals. (t(10)= 2.245; p< 0.05)
5.4 Discussion

The expression analysis of nine genes overexpressed in schizophrenia-related brain regions revealed that GABA$_A$$_delta$ has a tendency to be downregulated and wolframin is significantly underexpressed in the PCP model of schizophrenia (figures 5.6 and 5.10). Although the ISH study clearly showed that the expression of the majority of genes analysed is not modified by the PCP treatment, the interesting findings about GABA$_A$$_delta$ and wolframin are very encouraging, and support the validity of the strategy adopted in this project.

These results support the importance of the approach based on the anatomical specificity of the PCP-induced alterations, confirming that the extensive gene expression profiling obtained by LMD/microarray produced a database that can be a very useful source of information for research in schizophrenia and cognition.

5.4.1 GABA$_A$$_delta$

Some alterations of the GABAergic system in the PCP model of schizophrenia were demonstrated by Cochran et al. (2003) and include reduced expression of the calcium-binding protein parvalbumin in the prefrontal cortex and in the reticular thalamic nucleus (see section 1.3.4).

The present study suggests that the VO could also be specifically affected by the PCP-induced abnormalities of the GABAergic neurotransmission, as the GABA$_A$$_delta$ receptor showed a trend to be downregulated in this cortical subregion.

Although the change did not reach statistical significance (figure 5.6), the importance of the GABAergic neurotransmission for the neuropathology of
schizophrenia (reviewed by Lewis, 2000) and the previous findings on the PCP model confer interest to this finding.

Further investigations are needed to verify the validity of this result. Unfortunately the quantitative analysis of the ISH autoradiograms could not measure a statistically significant change, partly because the expression difference was very small, as commonly occurs in the brain. Nevertheless, this technique was very useful for investigating the expression of this gene in multiple specific brain regions; hence, increasing the number of biological replicates could be useful to increase the statistical power of the analysis without sacrificing the useful anatomical specificity of ISH.

5.4.2 Wolframin

Research on the wolframin gene in humans has been focused on the role of this gene in Wolfram syndrome, a complex neurological disease with symptoms including diabetes insipidus, diabetes mellitus, optic atrophy and deafness (see chapter 4).

Importantly, psychiatric illness is also a feature of Wolfram syndrome. Swift et al. (1990; 1991) emphasised this aspect of the disease reporting that a high proportion of Wolfram syndrome homozygotes had been hospitalised for mental illness, and committed or attempted suicide. Following these observations, it was hypothesised that the mutated wolframin could play a role in predisposing to psychiatric disease. In fact, it was reported that heterozygous carriers, who do not show the main Wolfram syndrome symptoms, are 26-fold more likely to suffer from psychiatric illness than non-carriers (Swift et al., 1998). The most consistent symptom self-reported by heterozygotes in this study was depression; suicide attempts, chronic anxiety, panic attacks and racing thoughts were also common.
These findings strongly suggest that wolframin plays a role in the predisposition to some forms of psychiatric diseases, and paved the way to more investigations. Although some studies failed to confirm the association between wolframin mutations and psychiatric diseases (Martorell et al., 2003; Kato et al., 2003); the increased tendency to suicide of the heterozygotes seems the most robust finding (Li et al., 2002; Sequeira et al., 2003).

For the first time, the present study showed that wolframin could be involved in the neuropathological alterations observed in a PCP model of schizophrenia. The ISH experiments measured a significant downregulation of wolframin in the PrL following the PCP chronic intermittent treatment. The consequences of this molecular abnormality are difficult to predict, partly due to the lack of knowledge on the biological functions of wolframin. Nevertheless, the reports that inactivating mutations of this gene predispose to some psychiatric symptoms in humans (Swift et al., 1991; Swift et al., 1998; Cryns et al., 2003) suggest that the underexpression of wolframin in the PCP model of schizophrenia could be part of the complex neurobiological changes underlying the behavioural abnormalities of the model. It is also worth considering that this neuropathological change was specifically measured in the PrL, a cortical subregion that controls crucial functions such as working memory and cognitions and that shows schizophrenia-related metabolic and molecular alterations (see above; Cochran et al., 2003).

Although the exact function of wolframin is unclear, evidence suggest that this gene may be involved in the regulation of calcium homeostasis, and could mediate the transport of this ion from the endoplasmic reticulum to the cytoplasm (see chapter 4). Calcium transport is finely regulated in excitable cells such as neurons, as changes of the Ca^{2+} concentration in the cytoplasm modulate important neurobiological processes including exocytosis. The Ca^{2+} concentration in the cytoplasm of neurons is regulated by a complex network of membrane channels, buffering proteins and proteins associated with the intracellular reservoirs. The glutamate NMDA receptor is a membrane Ca^{2+} channel that is directly involved in the neuropathology of schizophrenia, and blockers of this channel such as PCP can cause schizophrenia-like effects in humans and animals.
(see chapters 1 and 5). Parvalbumin, another protein that controls calcium homeostasis, is also involved in the neuropathology of schizophrenia and its expression is altered in the PrL of a PCP model (see above). Wolframin, that shows reduced expression in the PrL of the PCP model according to the present study, could be another gene involved in the complex mechanism that regulate calcium homeostasis in the prefrontal cortex that has a role in the biological basis of some symptoms of schizophrenia. Functional studies would be needed to verify this fascinating hypothesis. Moreover, it would be interesting to investigate the cellular localisation of wolframin to understand whether a specific subset of neurons of the PrL is affected by the PCP-induced alteration.
CHAPTER 6. EFFECT OF CHRONIC PCP TREATMENT ON EXPRESSION OF GENES ENRICHED IN THE PRL AND RT, THAT HAVE CHROMOSOMAL LOCATION LINKED TO SCHIZOPHRENIA
6.1 Introduction

6.1.1 Chromosomal loci linked to schizophrenia

It is now widely accepted that schizophrenia is a disease with a major genetic component, mainly arising from epidemiology evidence on families and twins (see section 1.5). In fact, it can be argued that schizophrenia has one of the highest heritabilities among the complex genetic disorders such as diabetes, cancer and coronary heart disease (Kirov et al., 2005).

This scenario has prompted a massive effort in trying to determine the specific genes that predispose a person to the disease. Such findings would be a tremendous step forward both for the understanding of the neuropathology of schizophrenia and for the development of novel treatments. However, this research has been as fascinating as daunting, because the complexity of the task has led in many cases to unclear or contradictory results (reviewed by Harrison et al., 2005; Kirov et al., 2005). It is clear that the heritability pattern of schizophrenia is very complex. There is no single gene or small cluster of genes that play a determinant role in the aetiology of the disease; in contrast, evidence suggest that multiple genes combined with epigenetic and environmental factors are likely to cause schizophrenia (see section 1.5).

Although some robust and convincing findings are reported in the literature (see below), there is still limited knowledge on how the schizophrenia-susceptibility genes interact with each other and with extra-genetic factors. Moreover, schizophrenia is not a homogeneous syndrome; in fact, patients diagnosed as schizophrenic can experience a different range of symptoms. This intrinsic variability makes the task of finding the "schizophrenia genes" even more difficult, as different molecular lesions could relate to different specific phenotypes. For these reasons the genome-wide linkage studies performed to date

195
report interesting but sometimes uncertain and contradictory data (reviewed by Harrison et al., 2005). However, two recent meta analyses have tried to clarify the useful information reported in the single studies.

Combining the data from 18 independent studies, Badner et al. (2002) concluded that three loci reached genome-wide significance: 8p, 13q and 22q. Lewis et al. (2003) used a different methodology to combine the data from 20 independent genome-wide scans. Only one locus, 2p, was found significant when stringent statistical criteria were applied, whereas the loci 5q, 3p, 11q, 2q, 1q, 22q, 8p, 6p, 20p and 14q were significant under less strict conditions.

Despite the discrepancy between the two studies, it is noteworthy that two loci, 8p and 22q, were significant in both. Arguably, the identification of schizophrenia-susceptibility genes cannot be achieved by the linkage analyses alone for the above-mentioned difficulties, but the findings need to be supported by expression studies and other biological evidence (Harrison et al., 2005).

In this scenario, it is remarkable that the gene encoding for the catechol-O-methyl transferase (COMT), which has been associated with schizophrenia by several independent studies (Harrison et al., 2005), maps to the chromosomal locus 22q that is significant in both Badner’s and Lewis’ meta analyses. Parvalbumin, which consistently showed reduced expression in the prefrontal cortex of schizophrenic brain and a PCP model of the disease (see chapter 4) also maps to the locus 22q. These observations support the suggestions that this chromosomal region should be considered as a strong candidate for schizophrenia susceptibility.
6.1.2 Localised expression of genes with chromosomal location linked to schizophrenia

As suggested by Harrison et al. (2005), the identification of schizophrenia susceptibility genes is not likely to come from linkage or association studies alone, but it requires additional biological evidence. In this scenario, it is interesting to consider the regional expression of genes that have a chromosomal location linked to schizophrenia. If such genes show a localised expression in brain regions involved in the neuropathology of schizophrenia, they could be interesting candidates for expression analysis in the schizophrenic brain. The same concept can be applied to animal models: the selection of candidate genes for expression studies can be based on the localised regional expression of genes whose human orthologs have a chromosomal location linked to schizophrenia.

The LMD/microarray study identified 42 genes overexpressed in the brain region analysed (PrL, VO and Rt), whose human orthologs have a chromosomal location corresponding to the schizophrenia sites identified by the meta analyses of Badner et al. (2002) and Lewis et al. (2003). The majority of those genes (28) were overexpressed in the Rt, whereas 12 showed localised expression in the PrL and only 2 in the VO (figure 6.1). These genes can be considered as potential candidates for expression analysis in the PCP model of schizophrenia. For technical reasons and time constraint, four of these 42 genes were selected for expression analysis after chronic PCP treatment (table 6.1).
Figure 6.1
Number of genes with schizophrenia-linked chromosomal location that show region-selective expression in the rat brain according to the LMD/microarray study.

<table>
<thead>
<tr>
<th>Regional selectivity</th>
<th>Gene</th>
<th>Chromosomal location</th>
<th>45mer probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrL</td>
<td>DPYSL3</td>
<td>5q</td>
<td>TCC ATC CCT TCA AAG ATG TTG TAT TCC GCA ACC GAC TGG TGG TTC</td>
</tr>
<tr>
<td></td>
<td>DPYSL5</td>
<td>2p</td>
<td>CAA AGC ACA GGG CCA TGT GTC GTC TCA GGA TTG ATA CAA CCT TAG</td>
</tr>
<tr>
<td>Rt</td>
<td>GPX3</td>
<td>5q</td>
<td>GGA GAA AGT GTG AAG GTG TTG TAC CAA ACC GTG AGC ACA GAT GGT</td>
</tr>
<tr>
<td></td>
<td>PIPPin</td>
<td>22q</td>
<td>CTG TGC TCA CAT TTT ACT TCC AGA CTG GGT CTC ATA AAG GTC ATT</td>
</tr>
</tbody>
</table>

Table 6.1
Genes with region-selective expression and chromosomal location linked to schizophrenia in the human genome that were selected for expression analysis in the PCP model of schizophrenia via \textit{in situ} hybridisation. (Refer to chapter 2 for details on design on the specific 45mer oligonucleotide probes).
6.1.3 Localised expression of genes with chromosomal location 5q

The locus 5q has been linked to schizophrenia by the meta analysis of Lewis et al. (2003) (see above). Interestingly, the LMD/microarray study showed that 11 genes that are overexpressed in brain regions relevant to schizophrenia are concentrated in this locus (figure 6.1).

Dihydropyrimidinase-like protein 3 (DPYSL3), also known as collapsin response-mediated protein-4, is one of the genes with location in 5q. The LMD/microarray study showed that the rat ortholog of this gene is overexpressed in the PrL. DPYSL3 is believed to play a role in neuronal differentiation, axonal outgrowth and possibly neuronal regeneration (Charrier et al., 2003). In fact, this gene belongs to the cluster of genes involved in neurodevelopment that are overexpressed in the PrL (see section 3.4.1).

Glutathione peroxidase 3 (Gpx3) is another gene with location in 5q. The rat ortholog of Gpx3 was overexpressed in the Rt according to the LMD/microarray study. The enzyme encoded by this gene is involved in the glutathione metabolism, and therefore in the protection of cells from oxidative stress. It has been shown that free radicals generation and oxidative stress may have a role in neuropsychiatric disorders (Ravikumar et al., 2000; Herken et al., 2001; Srivastava et al., 2001). Hence Gpx3 could be an interesting candidate for gene expression analysis in the PCP model of schizophrenia.

6.1.4 Localised expression of genes with chromosomal location 22q

The evidence that locus 22q is linked to schizophrenia is supported by both Badner’s and Lewis’ meta analyses; moreover, this is the genomic location of genes that show expression changes in the schizophrenic brain, such as COMT.
and parvalbumin (see above). The rat orthologs of 6 genes with genomic location in 22q are overexpressed in brain regions relevant to schizophrenia according to the LMD/microarray study; respectively, 4 are preferentially expressed in the Rt and 2 in the PrL.

One of the Rt selective genes encodes for PIPPin, a brain-specific mRNA-binding protein (Castiglia et al., 1996; Nastasi et al., 1999; Raimondi et al., 2003). This factor has recently attracted the attention of investigators for its role in the control of transcription. In fact, this protein specifically binds histone mRNAs, participating to the complex mechanisms regulating the transcriptional potential of the genome (Nastasi et al., 1999; Raimondi et al., 2003). The expression profile of PIPPin suggests that this gene could be important for the regulation of transcription in the Rt.

6.1.5 Localised expression of genes with chromosomal location 2p

2p is another interesting locus for schizophrenia, as it is the only one that achieved significance according to the most recent meta analysis when stringent statistical criteria were applied (see above; Lewis et al., 2003). The rat orthologs of 3 genes with genomic location in 2p are overexpressed in brain regions relevant to schizophrenia according to the LMD/microarray study; 2 are preferentially expressed in the Rt and 1 in the PrL.

The PrL selective gene with genomic location in 2p is Dihydropyrimidinase-like protein 5 (DPYSL5). Similarly to DPYSL3 (see above), this PrL selective transcript belongs to the collapsin response mediator proteins (CRMPs) family. These proteins are directly involved in apoptosis, proliferation, cell migration, and differentiation (see section 3.4.1; Charrier et al., 2003). Interestingly, two genes belonging to this family have a chromosomal location linked to schizophrenia and their rat orthologs are overexpressed in the PrL, a cortical subregion that is
directly involved in the cognition and attentional processes (see chapter 3). These observations suggest that DPYSL5 could be an interesting candidate for gene expression analysis in a rat model of schizophrenia.
6.2 Aims

The aim of this set of experiments was to analyse the gene expression of the rat orthologs of four genes with genomic location in schizophrenia-linked loci, after a chronic-PCP treatment regime that allows reproducing some aspects of schizophrenia in rats. The genes (DPYSL3, Gpx3, PIPPin and DPYSL5; see above) were selected on the basis of their expression profile in specific brain regions relevant to the neuropathological changes observed in the PCP model of schizophrenia.
6.3 Results

Genes overexpressed in the PrL

DPYSL3 and DPYSL5 showed overexpression in the PrL according to the LMD/microarray study (see table 3.3, “Neurogenesis/neurotransmission” functional class); therefore their expression level was analysed in this cortical subregion after chronic PCP treatment.

The quantitative analysis of the expression levels of DPYSL3 in the PrL of PCP-treated and control animals showed that there is no significant drug-induced expression change ($t(10) = -0.937; p>0.05$) (figure 6.2).

Similarly, DPYSL5 did not show significant expression change in the PrL of PCP-treated animals compared to vehicle treated controls ($t(10) = 1.134; p>0.05$) (figure 6.3).

Genes overexpressed in the Rt

The quantitative expression analysis of Gpx3 and PIPPin was performed in the Rt after chronic PCP treatment, as these genes are enriched in this region according to the LMD/microarray study (see table 3.2, “Neurotransmission” and “Transmission/RNA processing” functional classes).

The quantitative analysis of the expression levels of Gpx3 in the Rt of PCP-treated and control animals showed that there is no significant drug-induced expression change ($t(10) = 0.614; p>0.05$) (figure 6.4).

Likewise, the quantitative analysis of the expression levels of PIPPin in the Rt after chronic PCP treatment did not show a significant expression change ($t(10) = -0.183; p>0.05$) (figure 6.5).
Figure 6.2

A and B. In situ hybridisation (ISH) autoradiogram showing the expression of DPYSL3 at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of DPYSL3 expression in the PrL following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(10) = -0.937; p > 0.05).
Figure 6.3

A and B. In situ hybridisation (ISH) autoradiogram showing the expression of DPYS15 at the level of prefrontal cortex, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the prelimbic cortex (PrL) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of DPYS15 expression in the PrL following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(10) = 1.134; p>0.05).
**Figure 6.4**

*In situ* hybridisation (ISH) autoradiogram showing the expression of GPX3 at the level of anterior thalamus, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of RGS3 expression in the Rt following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals ($t(10)= 0.614; p>0.05$).
Figure 6.5

A and B. In situ hybridisation (ISH) autoradiogram showing the expression of PIPPin at the level of anterior thalamus, after chronic treatment with vehicle (saline) (A) and PCP (2.58 mg/kg) (B). The anatomical localisation of the reticular thalamic nucleus (Rt) is indicated by an arrow. Scale bar: 5 mm.

C. Quantification of PIPPin expression in the Rt following chronic PCP treatment by optical densitometry of ISH autoradiograms. No significant difference was measured between the PCP and vehicle treated animals (t(10)= -0.183; p>0.05).
6.4 Discussion

The expression analysis of four genes with chromosomal location linked to schizophrenia in the PrL and the Rt, following chronic PCP treatment, did not show drug-induced changes. The approach employed for this set of experiments was an attempt to combine the information from the recent comprehensive linkage meta analyses (Badner et al., 2002; Lewis et al., 2003) and the gene expression profiles obtained using the LMD/microarray strategy (see chapter 3) to choose candidate genes for expression analysis after a chronic PCP treatment regime that models some aspects of schizophrenia.

The negative results reported above illustrate the difficulty of individuating novel genes involved in the neuropathology of schizophrenia, especially when a small number of genes are investigated. However, the use of regional expression profiles for the choice of candidates has proved to be a valuable strategy (see chapter 5), therefore the combination of this information with linkage data may still have a potential usefulness, if the analysis is extended to a greater number of candidates.

A comprehensive expression analysis of all 42 region-selective genes with chromosomal location linked to schizophrenia would provide useful data, especially if performed using more than one technique, such as ISH and RT-qPCR. This type of study could be expanded to analyse these genes at the protein level. This approach is more likely to identify genes that show expression changes at the mRNA or protein level in the PCP model of schizophrenia; however, such a comprehensive scan is beyond the scope of this thesis mainly due to time constraint.

Another approach that could be used to verify if these genes are changed in the PCP model of schizophrenia is the combination of expression studies with behavioural tests. The expression levels of genes that are involved in cognitive and attentional processes in relevant brain regions such as the PrL and the VO
could be influenced by environmental factors and adaptation changes may occur in specific conditions, such as when performing complex cognitive tasks. In fact, unpublished data from our laboratories suggest that the expression of some genes are changed in animals that had been performing cognitive tasks compared to control animals that had been treated in identical manner, but did not perform behavioural task. In this scenario it is reasonable to hypothesise that some PCP-dependent expression changes may become apparent in some specific conditions only, such as during or soon after performing a cognitive task. Potential changes may only be revealed depending on the level of attentional load during cognitive testing in the model of schizophrenia. This hypothetical scenario could mirror some abnormalities measured by fMRI in the human schizophrenic brain, which are mainly observed when the cognitive functions are challenged by specific tests. This interesting but complex experimental strategy is also beyond the scope of this thesis, but could be an efficient way to use the data from the LMD/microarray study (see chapter 3) to perform a characterisation of the PCP model of schizophrenia after relevant cognitive tasks.
Chapter 7. General Discussion
The overall aims of this PhD thesis were to identify genes enriched in three brain regions (PrL, VO and Rt) implicated in some aspects of cognition and in some schizophrenia-like neurobiological alteration observed after a specific chronic PCP treatment regime in rats (Cochrane et al. 2003). Additionally, the aims of this thesis included the expression analysis of selected enriched genes following chronic PCP treatment.

These objectives were fully met, as the transcriptome profiling strategy based on the combination of laser-assisted microdissection and microarray analysis produced a novel and useful characterisation of the gene expression in the regions of interest. These data were employed to select candidate genes for expression analysis following chronic PCP treatment, allowing the detection of the downregulation of wolframin. This change may be a potentially important abnormality in the PrL of the PCP-treated rats with relevance to the human disease.

7.1 Expression profiling in laser-microdissected brain regions

The LMD/microarray study is the most innovative part of this thesis, and generated invaluable data that are the foundation of the research strategy employed to identify genes involved in the PCP-induced abnormalities (see chapters 5 and 6).

This ambitious project allowed the transcriptome profiling of the rat PrL, VO and Rt. The relevance of these specific regions to schizophrenia research is well documented, as they correspond to the structures affected by this psychiatric disease in the human brain (see chapter 3). Moreover they show crucial molecular and metabolic changes in a well-established model of schizophrenia that mirror
abnormalities observed in the schizophrenic brain (Cochran et al., 2003).

The combination of laser microdissection and microarray gene expression analysis is an innovative technique whose popularity is rapidly expanding in neuroscience research. This approach, which allows the identification of gene expression profiles in discrete structures of the brain, has recently been employed for the molecular characterisation of the hippocampus and the amygdala (Zirlinger et al., 2000; Bonaventure et al., 2002; Zirlinger, 2003; Datson et al., 2004; Torres-Munoz et al., 2004). In this study the LMD/microarray strategy was employed for the first time to investigate the gene expression profiles of subregions of the prefrontal cortex and one thalamic nucleus.

The results obtained are significant both from a technical and a biological perspective. In fact, in this study the LMD technique was optimised to identify and dissect specific cortical subregions with a high degree of reliability. The consistency of the technique was reflected by the uniformity of the data obtained from the biological replicates (see chapter 3).

The data obtained from the microarray analysis confirmed the hypothesis that the anatomical differences underlying the functional specificity of brain regions are associated with an unique gene expression profile. The differential gene expression between the Rt and the cortical regions examined was remarkable, and can be partly due to the gross anatomical differences and to the prevalence of GABAergic interneurons in this thalamic nucleus. Interestingly, the intra-cortical gene expression differences were also very prominent, despite the neuronal composition of the subregions examined being relatively uniform. The specific expression profiles of the PrL and VO identified in this study can be interpreted as the molecular substrate underlying the complex cognitive and attentional processes that require the activation of these cortical subregions.

The bioinformatic analysis and the information available from online databases (http://www.affymetrix.com, http://www.ncbi.nlm.nih.gov/UniGene, http://www.geneontology.org/, http://www.ensembl.org) highlighted the functional significance of some clusters of genes that were overexpressed in the regions analysed (see chapter 3). Arguably, the most intriguing finding produced
by this research is the overexpression of genes involved in neurodevelopment and neurogenesis in the PrL. The most plausible interpretation for the prevalence of such genes in the PrL (table 3.3) is their possible role in the mechanisms of plasticity that underlie cognitive and mnemonic processes. The involvement of neurodevelopment-related genes in cognition has been documented in the hippocampus, where neurotrophins such as BDNF, which is overexpressed in the PrL according to the present study, have an acute effect on synaptic transmission and regulate crucial processes such as long-term potentiation (Figurov et al., 1996; Patterson et al., 1996 Jovanovic et al., 2000). The finding that this class of genes is overexpressed in the PrL, a cortical subregion that controls working memory and behavioural flexibility, suggests that this expression pattern could be considered as a molecular fingerprint of areas involved in cognitive processes.

7.2 Confirmation of regional expression by in situ hybridisation

The results obtained from the validation study were crucial to confirm and to complement the microarray analysis. Although the modern oligonucleotides arrays are a very reliable tool for the exploration of gene expression, it is good practice to validate some of the data obtained with this technique using an alternative independent method such as ISH.

Remarkably, all transcripts that produced a detectable signal showed an expression profile corresponding to that predicted by the microarray analysis. The confirmation of the expression profiles of 11 genes supported the consistency not only of the microarray hybridisation and analysis, but also of upstream procedures such as the linear amplification. In fact, ISH allowed the quantification of the amount of transcript originally present in the brain regions of interest, as opposed
to the LMD/microarray strategy, which involved two steps of linear amplification (see chapter 3). The correspondence of the results obtained using these two strategies demonstrated that the linear amplification step had not altered the relative abundance of the 11 transcripts analysed in the different brain regions and therefore strongly support the reliability of this procedure.

The validation results also suggested that the LMD/microarray technique is more sensitive than ISH, as two of the 13 transcripts selected for validation (Dcx and Stk23, see section 4.4.1) could not be detected using the latter methodology. The increased sensitivity is another advantage of the microarray approach that can be very useful to investigate the expression of low-abundance genes.

Along with the invaluable confirmation data, the ISH experiments produced some useful information that complemented the expression profiles predicted by the LMD/microarray study for some of the 11 genes analysed. An example is the clarification of the laminar distribution of genes overexpressed in the PrL, such as FXYD6 and Wolframin. The ISH also highlighted that RGS3 and PKCδ, which were overexpressed in the Rt according to the LMD/microarray study, are expressed in the ventral part of this thalamic nucleus only. This expression pattern suggests that the ventral and the dorsal part of the Rt may have important functional differences in the rat brain.

7.3 Expression of validated localised genes in the chronic PCP model of schizophrenia

The research strategy of these experiments was based on the useful information obtained from the LMD/microarray and validation study. These data
allowed the selection of 9 genes for expression analysis following a chronic PCP treatment regime that models some aspects of schizophrenia in rats (Cochran et al., 2003).

Although the majority of these genes did not show significant expression changes in the brain regions of interest after the PCP treatment, a significant drug-induced downregulation of the gene wolframin was measured in the PrL. This interesting result supports the validity of the strategy employed and suggests that the regional expression profiles of the PrL, VO and Rt obtained via LCM/microarray can be invaluable for gene expression research in relation to psychiatric disorders and cognition.

Wolframin is a protein localised in the endoplasmic reticulum with a role in calcium transport (Takeda et al., 2001; Cryns et al., 2003; Osman et al., 2003) that has been linked to some forms of psychosis (Swift et al., 1998; Li et al., 2002; Sequeira et al., 2003). For the first time, the present study showed that this gene could be involved in the alterations observed in a PCP model of schizophrenia. The exact role of wolframin in these neuropathological processes is difficult to hypothesise due to the limited knowledge about its biological function. However, this gene, similar to NMDA receptors and parvalbumin, could be involved in the complex mechanisms that regulate calcium homeostasis in the PrL that have a role in the biological basis of some symptoms of schizophrenia.

7.4 Expression of genes with chromosomal location linked to schizophrenia in the chronic PCP model

In this section the selection criteria for the transcripts to analyse in the PCP model were based on the regional expression (LMD/microarray study) and the
genomic location of their human ortholog genes. This strategy combined the expression profiles in the PrL, VO and Rt obtained from the LMD/microarray study with the information from two recent meta analyses of schizophrenia linkage studies (Badner et al., 2002; Lewis et al., 2003) to designate four candidate genes. None of the candidates showed significant expression changes in the relevant brain regions in the PCP model of schizophrenia; however, the approach described above (see also chapter 6) could still be potentially useful if the analysis is extended to a greater number of candidates.

7.5 Further work

The interesting results reported in this thesis can inspire several fascinating questions. Above all, the gene expression profiles of the PrL, VO and Rt generated by the LMD/microarray study can be an invaluable source of information for gene expression research in the fields of molecular psychiatry and cognition.

These data could be integrated with protein expression studies. In fact, exploring the proteome of a specific brain region would add useful information and constitute a more complete "molecular fingerprint". Currently, the development of protein microarrays cannot match that of oligonucleotides array for reliability and sensitivity; therefore a proteomic profiling of brain regions would be unpractical. However, an analysis limited to a restricted number of genes of interest, would still produce interesting data and allow clarification of how the regional overexpression of some genes is reflected at the protein level.

Elucidating the role of neurodevelopment related genes overexpressed in the PrL would also be a fascinating task. Knowing how these genes (table 3.3)
participate to the neurophysiology of the PrL and how they interact with each other could be a major step forward in the understanding of the neurobiological mechanisms underlying cognition. These questions could be partly answered by performing behavioural and cognitive tests on knock out or knock down animals for the genes of interest. Also, it would be interesting to investigate whether the expression of neurodevelopment related genes in the PrL is modulated when rats perform tasks designed to assess different aspect of the executive function. In fact, unpublished data from our laboratories suggest that gene expression may be changed in the PFC of animals that had been performing cognitive tasks compared to control animals that had been treated in identical manner but did not perform the behavioural task.

Reduced expression of wolframin in the PrL of the model of schizophrenia is one of the most interesting finding reported in this thesis. The confirmation of this downregulation at the protein level would be a valuable goal to achieve. Wolframin specific antibodies have been successfully raised and employed in immunohistochemistry based experiments (Takeda et al., 2001; Ishihara et al., 2004; Philbrook et al., 2005); in fact immunofluorescence could be a suitable approach to investigate the expression of this protein in a specific brain structure such as the PrL. Moreover, co-localisation experiments could help clarifying whether wolframin is preferentially expressed in a specific subset of neurons of the PrL.

7.6 Conclusions

The present thesis reports a number of interesting findings relevant to cognition and modelling aspects of psychiatric disorders in rodents. The
combination of laser microdissection and microarray technology, an innovative approach that is becoming more and more common in neuroscience research, has been employed here for the first time to analyse the gene expression profiles of the rat PrL, VO and Rt. The data obtained represent original and useful information about the molecular basis of the functional differences in the specific brain regions investigated (see chapter 3).

Importantly, these findings were validated using an independent technique. The results obtained from the ISH confirmation study strongly supported the consistency and the reliability of the strategy employed for the transcriptome profiling. The ISH experiments also produced some interesting information that complemented the expression profiles predicted by the LMD/microarray study for some of the 11 genes analysed (see chapter 4).

The strategy adopted to investigate PCP-induced gene expression abnormalities (chapters 5 and 6) was based on the information obtained from the LMD/microarray study. This approach demonstrated that the chronic PCP treatment causes a downregulated of wolframin in the PrL of rats. This finding suggests that wolframin is directly involved in the neurobiological mechanisms that underlie the psychotomimetic effects of PCP, and could be potentially important in schizophrenia. This interesting original finding shows the potential utility of gene expression profiling of specific brain regions in neuroscience research. Further studies on the neurobiology of the proteins encoded by these genes may provide useful information for developing novel treatments for psychiatric disorders such as schizophrenia.
REFERENCES


Curr Opin Neurobiol, 6, 324-30.


Burnet, P. W., Eastwood, S. L. and Harrison, P. J. (1996) 5-HT1A and 5-HT2A receptor mRNAs and binding site densities are differentially altered in schizophrenia. *Neuropsychopharmacology*, 15, 442-55.


and arousal. Behav Brain Res, 9, 361-80.


Gao, X. M., Sakai, K., Roberts, R. C., Conley, R. R., Dean, B. and Tamminga, C. A. (2000) Ionotropic glutamate receptors and


Jentsch, J. D. and Roth, R. H. (1999) The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine...
hypothesis of schizophrenia. *Neuropsychopharmacology*, 20, 201-25.


Leveque, J. C., Macias, W., Rajadhyaksha, A., Carlson, R. R., Barczak,


Ron, M. A. and Harvey, I. (1990) The brain in schizophrenia. *J Neurol...
Neurosurg Psychiatry, 53, 725-6.


Seeman, P., Corbett, R. and Van Tol, H. H. (1997) Atypical neuroleptics have low affinity for dopamine D2 receptors or are selective for D4 receptors. Neuropsychopharmacology, 16, 93-110; discussion 111-35.


Shifman, S., Bronstein, M., Sternfeld, M., Pisante-Shalom, A., Lev-


Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas,


