IDENTIFICATION AND CHARACTERISATION OF NOVEL CANDIDATE GENES FOR SCHIZOPHRENIA

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- ABSTRACT -

Schizophrenia is a severe and debilitating mental disorder that affects 1% of the population. Clinical symptoms usually show at late adolescence or early adulthood and include a constellation of delusions, hallucinations, decreased motivation and impaired executive functions. The aetiology of schizophrenia remains unclear but like most psychiatric disorders it is classified as complex in origin, resulting from an interplay of genetic, developmental and environmental factors.

Recent intensive research has enabled the identification of susceptibility genes for schizophrenia, giving interesting insights into its pathophysiology. However, schizophrenia is such an heterogeneous and polygenic disease that more genes will have to be discovered, not only to help further towards a better understanding of the molecular mechanisms associated with schizophrenia but also to lead to the identification of novel pharmacological targets.

A global transcriptome screen to identify differentially expressed novel psychosis-related genes was performed utilising a phencyclidine (PCP) model of schizophrenia and rat oligonucleotide GeneChips from Affymetrix. The chronic PCP model of schizophrenia has been shown to produce a pattern of metabolic hypofunction and neurochemical changes in the rodent brain that closely mirror those observed in the brains of schizophrenic patients (Cochran *et al.* 2003). This microarray study allowed the screening of rat genes and expressed sequence tags (ESTs) that have the potential to represent novel and uncharacterised genes.

Bioinformatic analyses of the 209 significantly differentially expressed ESTs revealed 66 cDNAs. Three (*Edg2*, EST AI072720 and *Tm4sf12*) were selected as candidate genes for which to validate and characterise. Two of them, *Edg2* and EST AI072720, were confirmed as differentially expressed in the rat chronic PCP model by quantitative real-time PCR. The differential expression of *Tm4sf12* was not confirmed and therefore was not characterised further.

Edg2 (LPA1) is a G-protein-coupled receptor for lysophosphatidic acid (LPA), a mediator of diverse cellular activities. Is is widely expressed but predominantly in

the brain, where it was suggested to be involved in the control of myelination. *Edg2* distribution in the rat brain (as revealed by *in situ* hybridisation) was consistent with this hypothesis. However, its increased expression in the prefrontal cortex of rats after chronic treatment with PCP, consistently with increased expression in the dorso-lateral prefrontal cortex of schizophrenic patients, suggested that Edg2 may also have a role in cortical regions which may be related to schizophrenia. Microarray data from the rat chronic PCP model and from human post-mortem tissue led to hypothesise a novel pathway involved in schizophrenia that was termed the "Pyk/Nck" pathway. This signalling cascade leading from Ptk2b to activation of Jnk2 (Mapk9) is activated by NMDA receptor stimulation, therefore its suggested dysfunction is consistent with the antagonism properties of PCP at NMDA receptors. Edg2 activation was proposed to constitute another route to activating the "Pyk/Nck" pathway thus restoring NMDA receptor function in schizophrenia. Using a stable cell line overexpressing *EDG2* receptors, EDG2 activation was shown to induce gene expression changes within this pathway leading to an increased phosphorylation – hence activation- of Jnk2, its final output. These results provided *in vitro* evidence suggesting that compounds acting at the EDG2 receptor, via their ability to enhance activity within the "Pyk-Nck" cascade, may be promising for the treatment of schizophrenia. To enable screening of EDG2 agonists *in vitro*, a [^{35}S]-GTPγS binding assay was developed using the *EDG2*-overexpressing stable cell line. This assay was shown to be sensitive enough for being used as a high throughput screening assay.

Using techniques that enable the analysis of transcripts (RACE and RT-PCR), the EST AI072720 was confirmed as a genuine rat transcript, which had been hypothesised from bioinformatics analyses. "Similar to KIAA1189 protein" (*RGD1308367*), is an uncharacterised rat gene predicted to have a C-terminal ezrin-radixin-moesin (ERM) actin-binding domain which suggests it may have a role in cytoskeletal architecture.

Differential expression of *RGD1308367* was confirmed in the rat chronic PCP model by quantitative real-time PCR, while its human orthologue *KIAA1189* also showed a significant increase in post-mortem tissue from schizophrenic patients. As expected, *in situ* hybridisation on EST AI072720 and *RGD1308367* revealed the same expression pattern in the rat brain, with very high expression within all major white

matter tracts and much lower expression within all cortical and sub-cortical areas. FLAG immunofluorescence was performed using C6 rat glioma cells overexpressing a FLAG-KIAA1189 fusion protein, to determine the sub-cellular localisation of the protein. KIAA1189 was found to be expressed in the plasma membrane and in neuronal-like processes and colocalisation with actin (suggested from the predicted C-terminal ERM domain) was confirmed by double labelling with phalloidin, a toxin that specifically binds F-actin. Because actin filaments are known to play a crucial role in neurite extension, a potential role for KIAA1189 in neurite outgrowth was investigated *in vitro* using PC12 cells overexpressing KIAA1189 and undergoing NGF-induced differentiation. Using a semi-quantitative assay, overexpression of a FLAG-KIAA1189 protein was found to prevent neurite outgrowth by comparison with the empty FLAG expression vector. Although this role will have to be further characterised, such a function of KIAA1189 may be consistent with the major theories of schizophrenia, the overexpression of KIAA1189 at critical times of development potentially affecting neuronal connectivity while its overexpression following chronic PCP treatment may be related to altered synaptic plasticity by modulating cytoskeletal changes of dendritic spines in response to learning and experience.

Finally, genotyping of three single nucleotide polymorphisms (SNPs) within the human *KIAA1189* gene did not reveal any significant genetic association with schizophrenia in a population of 600 schizophrenic patients and controls. However, this transcript maps to chromosome 2q, a key schizophrenia locus pointed out in several linkage studies and recently associated with visual working memory. This genetic evidence adds to our functional evidence suggesting that *KIAA1189* may be an intriguing novel candidate gene for schizophrenia.

All together, this work has identified and characterised two interesting candidate genes for schizophrenia. One of them, *EDG2*, may represent a promising drug target for schizophrenia while the other one, *KIAA1189*, may provide novel insights into the pathophysiology of schizophrenia and further evidence supporting its neurodevelopmental hypothesis.

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ac	anterior commisure
acg	anterior cingulate cortex
aci	intrabulbar part of the anterior commisure
AEBSF	[4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride]
ANCOVA	analysis of covariance
ASST	attentional set-shifting task
ATP	adenosine triphosphate
Au1	primary auditory cortex
BGH	bovine growth hormone
BLAST	basic local alignment search tool
BSA	bovine serum albumin
°C	degree Celsius
CA1pcl	pyramidal cell layers of the CA1 region of the hippocampus
CA2pcl	pyramidal cell layers of the CA2 region of the hippocampus
CA3pcl	pyramidal cell layers of the CA3 region of the hippocampus
cAMP	adenosine 3'-5' cyclic monophosphate
cc	corpus callosum
CCD	charge coupled device
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
cg	cingulum
CMV	cytomegalovirus
CNS	central nervous system
CRF	corticotropin releasing factor
DAPI	4',6-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
DEPC	diethylpyrocarbonate
DGgcl	dentate gyrus granule cell layer
DLPFC	dorso-lateral prefrontal cortical
D-MEM	Dulbecco's modified eagle medium

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DP	Dynamic Programming
DPM	disintergrations per minute
ds	double stranded
DSM-IV	Diagnostic and Statistical Manual IV
DTT	dithiothreitol
E-64	[N-(trans-Epoxysuccinyl)-L-leucine 4- guanidinobutylamide]
EBI	European Bioinformatics Institute
ec	external capsule
EC_{50}	effective concentration 50%
ECACC	European Collection of Cell Cultures
ECL	enhanced chemiluminscence
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalography
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPS	extrapyramidal symptoms
ERM	ezrin-radixin-moesin
EST	expressed sequenced tag
f	fornix
FAM	6-carboxyfluorescein
FBS	foetal bovine serum
FDA	Food and drug Administration
FDR	false discovery rate
fi	fimbria of hippocampus
fmi	forceps minor corpus callosum
fmj	forceps major corpus callosum
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
gcc	genu of corpsu callosum
gDNA	genomic deoxyribonucleic acid

GDP	guanosine diphosphate
GFP	green fluorescent protein
GIP	G protein coupled receptor-interacting protein
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
HEPES	(N-[2-hydroxyethylpiperazine-N'-2[2-ethanesulfonic acid]]
HMM	Hidden Markov Models
hnRNA	heterogeneous nuclear ribonucleic acid
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
ic	internal capsule
ICD-10	International Classification of Diagnosis
IgG	immunoglobulin G
iL	infralimbic cortex
i.p.	*intra peritoneous*
kb	kilobase
kDa	kilo Dalton
LB	Luria-Bertani
lent	lateral entorhinal cortex
ln	neperian logarithm
lO	lateral orbital cortex
log	logarithm
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LSD	lysergic acid
M1 and M2	primary and secondary motor cortices
MAM	methylazoxymethanol acetate
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MATRICS	Measurement and Treatment Research in Cognition in Schizophrenia
MCS	multiple cloning site
MIAME	minimal information about a microarray experiment
miRNA	microRNA
MMDB	Molecular Modeling Database

MPSS	massively parallel signature sequencing
MR	maximum resolution
mRNA	messenger ribonucleic acid
mt	mammillothalamic tract
NAA	N-acetyl aspartate
NAAG	N-acetylaspartylglutamate
NCBI	National Center for Biotechnology
NGF	nerve growth factor
NIMH	National Institute of Mental Health
NMDA	N-methyl-D-aspartic acid
NN	Neural Networks
OD	optical density
oligo-dT	oligo-(deoxythymidylic acid)
OMIM	Online Mendelian Inheritance in Man
ORF	open reading frame
PA	phosphatidic acid
PBS	phosphate buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PFC	prefrontal cortex
PMSF	phenylmethylsulphonyl fluoride
PPI	pre-pulse inhibition (of startle)
PrL	prelimbic cortex
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative real-time reverse transcriptase polymerase chain reaction
RACE PCR	rapid amplification of cDNA ends polymerase chain reaction
RGSPC	rat genome sequencing project consortium
RIPA	radioimmunoprecipitation assay
RMA	Rrobust Multichip Average
RNA	ribonucleic acid
RNAse	ribonuclease
rsa	agranular retrosplenial cortex
rsg	granular retrosplenial cortex

RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SAGE	serial analysis of gene expression
SAM	Significance Analysis of Microarrays
SBH	subtraction by hybridisation
SDS	sodium dodecyl sulphate
SDS-PA	sodium dodecyl sulphate polyacrylamide
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
sem	standard error of the mean
sm	stria medullaris of the thalamus
SNP	single nucleotide polymorphism
SOB	super optimal broth
SOC	derivative of SOB medium containing glucose that gives rise to catabolite repression
SSC	Sodium chloride sodium citric acid
TAMRA	6-carboxytetramethylrhodamine
TBE	tris-borate EDTA
TBS	tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
TE	tris EDTA
TIGR	Institute of Genome Research
Tm	melting temperature
TMB	tetramethylbenzidine
TOGA	total gene expression analysis
Tris	tris(hydroxymethyl)amino methane
TURNS	Treatment Units for Research on Neurocognition in Schizophrenia
UCLA	University of California Los Angeles
UTR	untranslated region
UV	ultraviolet
VIC	trademark product from Applied Biosystems
vO	ventral orbital cortex
vs	*versus*
WHO	World Health Organisation

WTSI	Wellcome Trust Sanger Institute
3D	3-dimensional
5-HT	serotonin
6-OHDA	6-hydroxydopamine

CHAPTER 1: INTRODUCTION

1.1- Schizophrenia

Schizophrenia is a chronic, severe and debilitating mental disorder characterised by profound disturbances of cognition, emotion and social functioning. The lifetime risk of developing schizophrenia is approximately 1% in the general population, with a typical onset during adolescence or early adulthood (Andreasen 2000; Sawa and Snyder 2002).

Schizophrenia is one of the most important public health concerns for society, not only because of its costs but also because it damages some of the most advanced functions of the brain that we regard as specifically human (Wong and Van Tol 2003).

Although extensive research over the past half century has allowed us to improve our knowledge of schizophrenia, it still only provides an incomplete understanding of this complex and multifactorial disorder which results from an interplay of genetic and environmental factors (Sawa and Snyder 2002). Similarly, despite improvements in the tolerability and, to some extent, in the efficacy of schizophrenia pharmacotherapy, new treatments still follow approximately the same paradigm as older antipsychotics, which may explain their failure to treat effectively many refractory patients (Miyamoto *et al.* 2005). Therefore it remains of major importance to improve both the understanding of schizophrenia pathophysiology and its pharmacotherapy.

In this context, novel insights may come in particular from translational approaches aiming at establishing a link between mechanistic hypotheses of the disease in animals and the complex disorder of cognition and behaviour that schizophrenia represents in human.

1.1.1- Diagnosis, symptoms and treatment of schizophrenia

1.1.1.1- Diagnosis and symptoms of schizophrenia

In addition to having a complex multifactorial aetiology, schizophrenia is also complex in that it affects nearly all domains of mental functions, with diverse and variably expressed symptoms including disorganised thought patterns, delusional beliefs, hallucinations, blunted or incongruous mood, apathy and social withdrawal. Nevertheless, since not any molecular abnormality seems to be common to all patients with schizophrenia, its diagnosis is based entirely on clinical symptoms, and operational explicit criteria associated with structured interviews have been defined to achieve a high degree of diagnostic reliability (Bray and Owen 2001).

The criteria for the diagnosis of schizophrenia are currently defined in the 4th edition of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV, (American Psychiatric Association 1994) and the 10th edition of the International Classification of Diagnosis (ICD-10, (WHO 1992). Both classifications establish the diagnosis of schizophrenia on the prominent presence of particular symptoms from specified sets of symptoms for defined periods of time. Thus, while the ICD-10 demands that symptoms from a set of characteristic symptoms are clearly present for the majority of time for at least 1 month, the DSM-IV requires that characteristic symptoms are present for 1 month but also that one of these symptoms persists for more than 6 months.

The characteristics of schizophrenia are frequently classified into positive and negative symptoms and cognitive dysfunction (Bassett *et al.* 1993; Andreasen *et al.* 1994).

Positive symptoms include hallucinations (sensory experiences in the absence of external stimulation), delusions (irrational beliefs that are unresponsive to logical argument) and thought disorder:

- Hallucinations may be visual, or even, occasionally, olfactory or tactile, but they are most often auditory hallucinations of human speech, "voices" which may

comment on the patient's actions or thoughts or more dramatically command the patient to action.

- Delusions typical to schizophrenia are commonly referred to as paranoid, and include delusions of persecution, grandiosity, external control, having thoughts inserted or withdrawn from one's head, ideas of reference and mind reading.
- Thought disorder may be described along a spectrum of severity ranging from tangential and circumstantial thinking to loosening of associations and disorganised speech.

Negative symptoms may be described as a loss of capabilities that most well people have. They include severe disturbances in social interaction, motivation, expression of affect (flattened or blunted affect and impaired emotions), spontaneous speech (alogia), ability to seek out and experience pleasure (anhedonia) and a fall in self-esteem leading to personal neglect (avolition-apathy) (Hales 1994). In extreme cases, people with schizophrenia may become almost totally unresponsive and will not move, speak or respond (a condition known as catatonia).

Negative symptoms may coexist with positive symptoms from the outset (primary negative symptoms) or appear later in the illness (secondary negative symptoms), sometimes as a side effect of drug treatment (Andreasen *et al.* 1994). Moreover, unlike positive symptoms, negative symptoms typically do not respond well to current antipsychotic medication.

Cognitive symptoms are probably the most important symptoms of schizophrenia as they relate to disturbances of logical thought processes that are required for a number of mental activities and are exhibited by the large majority of schizophrenic patients (even those regarded as "almost well") (Kelly *et al.* 2000). They include deficits in attention and memory (both verbal and non-verbal) and executive function, including the ability to plan, weigh-up situations, form abstract concepts and take appropriate courses of action. In particular, working memory may be affected, which is important because it helps to keep information in the mind and use it in complex decisions. Many patients demonstrate cognitive impairment prior to clinical symptoms (Jones *et al.* 1994) and some deficits may also be detected in an attenuated form in non-schizophrenic relatives (Cannon *et al.* 1994) suggesting that these deficits are an inherent biological phenomenon rather than a correlate of chronic antipsychotic

medication. Cognitive deficits have a major impact on many aspects of daily life of schizophrenic patients, compromising social function, education and employment (Weickert *et al.* 2000) and have the most substantial impact upon illness outcome in schizophrenia, more than positive or negative symptoms (Green 1996).

Although schizophrenia has a typical onset during adolescence or early adulthood (Andreasen 1995; Sawa and Snyder 2002), the disease can follow different courses which again reflect its complexity and its heterogeneity. Thus, it may start with an acute psychotic episode expressed most commonly by hallucinations, delusions and formal thought disorder, or develop gradually through progressive changes in behaviour. Acute schizophrenia may disappear and never come back; in fact, almost 25% of people diagnosed with schizophrenia recover completely within 5 years. However, the first episode is usually followed by a series of intermittent episodes of psychotic symptoms on top of a persistent disability with pronounced negative symptoms and cognitive deficits (Watt *et al.* 1983). In chronic-onset schizophrenia, symptoms are usually hardly visible at first but develop gradually and are less often followed by acute episodes. The long term is variable. About 10% of schizophrenic patients suffer from persistent, severe disability throughout life, while among patients who suffer from severe illness in the early phase of the disease over 50% function quite well in later life. It has also been shown that patients receiving treatment soon after the beginning of the disease usually have a better outcome (Johnstone *et al.* 1986).

Overall, although symptoms of schizophrenia vary in intensity over time, the negative and cognitive dysfunction are quite persistent whereas chronic psychotic symptoms have an episodic pattern (Andreasen 1995). Therefore it really makes sense that none of these symptoms alone are diagnostic for schizophrenia and in fact, the criteria defined by the ICD-10 and the DSM-IV have been structured to allow for the possibility that two patients with schizophrenia may not overlap in their symptomatology (Wong and Van Tol 2003). Thus, the question of what clinical deficit is central to the disease remains unanswered and clinically, three major types of schizophrenia have been proposed: among them, paranoid schizophrenia (including hallucinations and delusions) is by far the most common followed by hebephrenic (characterised by unpredictable behaviour and incoherent speech) and

catatonic (with stupor and unresponsiveness alternating with states of high excitement) schizophrenia (ABPI 2003; Wong and Van Tol 2003). However, there are no correlations between these sub-classes and response to treatment or other outcomes (Marneros *et al.* 1995).

Therefore, the diversity of schizophrenia, together with its unknown molecular aetiology, makes it difficult -if not impossible- to find a treatment which may improve all the different features of the disease.

1.1.1.2- Schizophrenia medication

It was only about 50 years after the word "schizophrenia" was introduced by E. Bleuler (1911), replacing the "dementia praecox" described by E. Kraepelin, that the first effective treatment for schizophrenia, chlorpromazine, was made available. Since then, development of medicines and understanding of the functions of the brain and of the neurochemical basis of schizophrenia have progressed side by side, characterisation of the pharmacological profile of some compounds yielding insights into the involvement of neurotransmitters in schizophrenia while such findings stimulated the search for and the synthesis of further compounds with specific properties. A number of medicines were thus developed and antispychotics of various chemical classes have been used in the treatment of schizophrenia for over 40 years.

"Typical" (or "conventional") antipsychotic drugs such as haloperidol, perphenazine, trifluoperazine and fluphenazine have been the first line of schizophrenia therapy since the introduction of chlorpromazine in 1954. The main therapeutic mechanism of these drugs lies in their ability to block dopamine D2 receptors, which probably mediates their effectiveness in controlling positive symptoms such as hallucinations and delusions (van Rossum 1966). However, these drugs do not improve the negative symptoms and the cognitive deficits associated with schizophrenia. Moreover, blockade of dopamine receptors in the striatum appears to be responsible for motor side effects termed extrapyramidal symptoms (EPS) which resemble motor abnormalities (tremors, akinesia, *i.e.* slowing of voluntary movements, rigidity and akathisia, *i.e.* discomfort in the legs and inability to sit for long periods of time) observed in Parkinson's disease (Meltzer and Stahl 1976; Carlsson 1978; Peacock *et*

al. 1996). Tardive dyskinesia, a choreathetotic movement disorder causing uncontrollable movements, initially of the face, lips and tongue but eventually affecting other parts of the body, is the most serious extrapyramidal symptom since it develops, generally after several years of treatment, in about 20% of patients treated with typical antipsychotics and is most often irreversible (Gardos *et al.* 1994). Moreover, a significant proportion of schizophrenic patients do not respond or become resistant to typical antipsychotic drugs during their illness (Mayerhoff *et al.* 1994). However, the most important drawback of these drugs is their inability to treat the negative symptoms and improve cognitive dysfunction, resulting in chronic morbidity and poor long-term outcome.

A second generation of antipsychotic drugs termed "atypical" antipsychotics was thus developed following on from the synthesis of clozapine and the realisation of the potential of this drug many years later in clinical trials. Clozapine, which was notably almost devoid of extrapyramidal (EPS) side effects, proved indeed to display an improved efficacy against the negative symptoms and, most importantly, against the cognitive dysfunction of schizophrenia and was interestingly effective in a proportion of patients resistant to older antipsychotic treatments (Brunello *et al.* 1995). Unfortunately, it was soon realised that clozapine caused a very severe agranulocytosis in a small number of patients and clozapine was immediately withdrawn from the market in 1975. Finally, after more rigorous clinical trials, it was reintroduced and is now used in refractory patients with regular blood monitoring under the control of a specialist. Clozapine's atypical pharmacological properties have been attributed to its unique pharmacological profile, which includes a lower affinity for dopamine D2 receptors but a notable affinity for serotonin, adrenergic, dopamine, histamine and muscarinic receptors (Tamminga 1997). Therefore, attempting to resemble the profile of clozapine, a number of other "atypical" antipsychotics were developed in which blockade of dopamine D2 receptors was no longer the sole therapeutic mechanism (Meltzer 1989). Thus, these second-generation antipsychotics, including risperidone, olanzapine, quetiapine and ziprasidone share the dopamine-serotonin antagonism of clozapine (Freedman 2003) but also display unique pharmacological profiles with diverse affinities for other neuroreceptors including muscarinic cholinergic and histamine receptors and potential interactions with the glutamate system (Miyamoto *et al.* 2005).

With regards to efficacy, most second-generation antipsychotics have been shown to offer some advantages over the "typical" antipsychotics particularly through their ability to improve the negative symptoms (Moller 2003) and the cognitive impairment of schizophrenia to some extent (Meltzer and McGurk 1999; Harvey and Keefe 2001) and prevent potential relapse (Miyamoto *et al.* 2005). However, concerns about extrapyramidal (EPS) side effects of first-generation antipsychotics have been replaced by other distressing effects including weight gain, hyperglycemia and dyslipidemia (Volavka *et al.* 2002; Baptista *et al.* 2004; Newcomer 2005).
The therapeutic benefit of "atypical" antipsychotics is therefore controversial and their advantages are most often regarded as not necessarily substantial (Miyamoto *et al.* 2005) especially since the extent to which they improve cognition in schizophrenic patients may not translate into an improved quality of life (Cramer *et al.* 2001).

These limitations of existing antipsychotic drugs have made people aware that no single treatment may be sufficient, paving the way to the search for compounds to be used in adjunction to antipsychotic drugs. Various adjunctive treatments including benzodiazepines, lithium, anticonvulsants, antidepressants, beta-blockers and dopamine agonists, have thus been used to treat residual symptoms of schizophrenia or to enhance the response to antipsychotic medications (Miyamoto 2002). However, theoretical and regulatory rationale to support this practice only came recently with the recognition of cognitive impairment as a critical and independent feature of schizophrenia that needs to be targeted independently (Friedman *et al.* 1999; Gold 2004). This is reflected by two recent initiatives of the American National Institute of Mental Health (NIMH): Measurement and Treatment Research in Cognition in Schizophrenia (MATRICS, (Marder and Fenton 2004) and Treatment Units for Research on Neurocognition in Schizophrenia (TURNS). In addition, as a consequence of the development of a battery of neurocognitive tests by the MATRICS consortium, the Food and Drug Administration (FDA) has now made it clear that it is reasonable for industry to seek approval for medications that enhance cognition in schizophrenia, which marks an important turning point in schizophrenia therapeutics (Gold 2004).

The development of novel additional strategies to obtain potentially new antipsychotic compounds and adjuncts possessing unique pharmacological profiles with fewer side effects is still a major challenge, both scientifically and financially (Miyamoto *et al.* 2005). However, this challenge is actively being pursued by the pharmaceutical industry based on specific hypotheses, and a number of agents with antipsychotic efficacy are currently being developed. In addition, novel approaches aiming at tailoring treatments or treatment regimes based on particular genotypes, SNP profiles or any other technique defining subgroups of patients are also pursued that have the potential to maximise the efficacy of the drug and to minimise the risk of developing side effects.

1.1.2- Genetic and environmental basis of schizophrenia

Although the prevalence of schizophrenia in the general population is about 1%, the results of numerous family, twin and association studies have conclusively shown that the risk of developing this illness is increased among the relatives of affected individuals, suggesting that genetic factors are largely involved (Gottesman 1991; McGuffin 1994; Riley 2004). The five most recent twin studies report a concordance rate among monozygotic twins of 41-65% compared to 0-28% for heterozygotic twins, estimating the broad heritability (*i.e.* the proportion of the total variance of the phenotype "schizophrenia" that is accounted for by genetic effects) of schizophrenia of about 85% (Cardno and Gottesman 2000). Thus, the approximate 10-fold increase in risk of developing schizophrenia in children and siblings of individuals with schizophrenia may be explained by a reduction in the reproductive opportunities, drive and possibly the fertility of affected individuals (Kirov *et al.* 2005).

Nevertheless, while twin and adoption studies clearly show that genetic factors are essential, they also point out the importance of environmental factors since the concordance for schizophrenia in monozygotic twins is typically around 50% and heritability is less than 100% (Owen *et al.* 2005a). Thus, it appears that what is inherited is probably the predisposition to develop the disorder, rather than the certainty of disease accompanying a genotype (Jurewicz *et al.* 2001). In other words, schizophrenia appears largely genetically "mediated" but not genetically "determined" (Riley and Kendler 2006). Moreover, there are recent studies

suggesting that gene-environment interactions may also be important components in the overall risk (Tienari *et al.* 2004).

Studies of the relative risk in various relatives have excluded the possibility that schizophrenia is a single-gene disorder or collection of single-gene disorders even if these genes had an incomplete penetrance (Jurewicz *et al.* 2001). Rather, they strongly suggest that, similar to other common disorders, schizophrenia has a complex and non-Mendelian mode of transmission (Gottesman and Shields 1967; McGue and Gottesman 1989). However, the number of susceptibility genes (oligogenic and polygenic modes of transmission may be possible), the disease risk conferred by each locus and the degree of interaction between loci remain unknown (Kirov *et al.* 2005). Risch (1990) has calculated that the data for recurrence risks in the relatives of probands with schizophrenia are incompatible with the existence of a single locus conferring a relative risk in siblings (*i.e.* a risk in siblings relative to the risk in the general population) of more than 3 and that, unless there is extreme interaction between genes (a phenomenon called epistasis), models with two or three loci of relative risks less than or equal to 2 are more plausible (Risch 1990). However, this does not exclude that genes of larger effects may exist in some subpopulations of patients or in families with a high density of illness since such families are normally expected under a model of polygenic inheritance (McGue and Gottesman 1989).

In summary, studies of the inheritance of schizophrenia have thus revealed that schizophrenia is a multifactorial disease characterised by the contribution of multiple susceptibility genes that probably act in conjunction with epigenetic processes and environmental factors.

1.1.3- Neurochemical basis of schizophrenia

Attempts to elucidate the biological correlates of schizophrenia have traditionally focused on several levels of explanation, including the neuropsychological, the neuropathological and the neurochemical evidence. The theory according to which schizophrenia is caused by disturbances in particular neurotransmitter systems arose during the 1950s and 1960s, based on the lack of obvious structural abnormalities in

the schizophrenic brain and motivated by the observed antipsychotic or psychomimetic effects of certain chemical compounds. Thus, the dopamine hypothesis (van Rossum 1966; Carlsson 1978), which suggests that schizophrenia is associated with a hyperdopaminergic state, was proposed based on the proportional correlation observed between the potency of the first generation of antipsychotic drugs and their ability to block dopamine receptors (Seeman and Lee 1975) (section 1.1.1.2). This theory has long prevailed as the dominant neurochemical theory of schizophrenia before a more complex picture was suggested whereby the increased dopaminergic transmission in the basal ganglia is associated with acute psychosis (Abi-Dargham *et al.* 2000) and the chronic prefrontal cortical deficit is responsible for cognitive impairments (Weinberger *et al.* 2001) resistant to first-generation antipsychotic drugs. According to this refined hypothesis, which was notably suggested by the understanding of the pharmacological profile of clozapine (section 1.1.1.2), the clinical efficacy of recent antipsychotics was proposed to be partly mediated by actions on other neurotransmitter systems in addition to dopamine, which is consistent with evidence showing alterations in serotoninergic (Woolley and Shaw 1954), glutamatergic (Kim *et al.* 1980) and GABAergic (Roberts 1972) neurotransmission in schizophrenia.

Evidence suggesting that a dysfunction in glutamatergic neurotransmission might be involved in the pathophysiology of schizophrenia has been accumulating in the last two decades mainly based on the observation that NMDA (N-methyl-D-aspartic acid) glutamate receptor antagonists such as phencyclidine (PCP) and ketamine can cause psychotic symptoms in normal humans (Malhotra *et al.* 1996) and exacerbate these symptoms in schizophrenic patients (Javitt and Zukin 1991; Malhotra *et al.* 1997b). Moreover, adjunctive treatment with NMDA agonists such as glycine has been shown to provide a modest improvement of negative symptoms in schizophrenia (Javitt *et al.* 1994; Heresco-Levy *et al.* 1999). Interestingly, extensive interactions of the glutamatergic system with other neurotransmitter systems suggest that alterations in glutamate may affect other systems that may be implicated in schizophrenia so that the glutamate hypothesis of schizophrenia cannot be considered separately. Thus, schizophrenia may result from an alteration of the delicate balance between dopaminergic and glutamatergic neurotransmission within critical regions of the brain (Holcomb *et al.* 2004). Evidence supporting this theory includes data

showing that glutamate neurons regulate the function of GABA (Gamma-aminobutyric acid) interneurons (whose morphology has been shown to be altered in schizophrenia; Lewis *et al.* 2005) and dopamine neurons (which are the target of antipsychotic drugs). In addition, NMDA receptor function appears to be strongly related with serotoninergic activity (Breese *et al.* 2002) while glutamatergic neurotransmission can be affected by neuropeptide modulators such as cholecystokinin (CCK) and N-acetylaspartylglutamate (NAAG), that have been shown to be altered in schizophrenia (Meador-Woodruff and Kleinman 2002).

Whether alterations in glutamatergic neurotransmission, like any other neurochemical findings, reflect primary rather than secondary, downstream pathology, compensatory mechanism or environmental influences remains unclear (Bray and Owen 2001). However, the fact that reciprocal connections between key cortical areas are exclusively glutamatergic suggests that glutamatergic neurons may represent the means by which aberrant information is transferred within and between different regions of the brain (Moghaddam 2003). Moreover, there is accumulating evidence that NMDA glumatate receptors may be critical in triggering the alteration, in the schizophrenic brain, of limbic thalamocortical circuits that integrate the roles of glutamate, dopamine and GABA and therefore are consistent with current hyperdopamine and hypoglutamate theories of schizophrenia (Tamminga 1999; Carlsson *et al.* 2000; Sharp *et al.* 2001; Coyle 2004).

1.1.4- Neuropathology of schizophrenia

A number of neuropathological abnormalities have been reported in the brains of schizophrenic patients, including reduced cortical volume, smaller glutamatergic somatic or neuropil size, reduced synaptic protein expression, disarray of neuronal orientation and area- and lamina-specific reductions in dendritic field size and dendritic spines. These abnormalities, which concern a number of regions (including the prefrontal cortex, the hippocampus and adjacent entorhinal cortex, the thalamus) rather than a few specific brain regions, are thought to represent the basis for at least a proportion of the aberrant connectivity (Harrison 1999). Besides, some of these post-mortem findings have interestingly been paralleled by *in vivo* structural

neuroimaging findings indicating reduced cortical volumes, magnetic resonance spectroscopy studies consistent with reduced neuropil volume or viability and diffusion tensor imaging studies suggesting disturbances in cortical connectivity (Krystal *et al.* 2003).

A novel approach to understanding the pathophysiology of schizophrenia has thus emerged over the last decade based on the understanding that the complex brain functions disturbed in schizophrenia are dependent on the coordinated activity of distributed ensembles of neurons (Lewis 2002).

1.1.4.1- The dorso-lateral prefrontal cortical (DLPFC) circuit in schizophrenia

Studies examining potential alterations in neural circuits rather than specific brain regions were performed, and the dysfunction of the corticolimbothalamic circuit emerged as having the potential to correlate alterations in multiple neurotransmitter systems and observed brain abnormalities with the clinical symptoms, especially the cognitive dysfunction, of schizophrenia (Bunney and Bunney 2000; Lewis 2002; Morris *et al.* 2005). Within this circuit, the Dorso-Lateral Prefrontal Cortex (DLPFC), because of its integrative role, was suggested to represent the essential region. The DLPFC circuit then runs from the prefrontal cortex to the ventral striatum on to the ventral pallidum then to the mediodorsal thalamic nucleus before returning to the prefrontal cortex.

Evidence suggesting that this circuit was compromised was found at the level of anatomical, neuropathological and transmitter-related molecules and defects in any of the structures that it connects were shown to produce alterations potentially related to schizophrenic symptoms (Bunney and Bunney 2000). Moreover, imaging of regional cerebral blood flow and metabolic activity in schizophrenic patients interestingly revealed disturbances in the activity of the DLPFC circuit, with lower activity in the prefrontal, cingulate and temporal (including the hippocampus) cortical areas, the striatum/accumbens region and the thalamus (Hazlett *et al.* 2004; Morris *et al.* 2005). In particular, robust and specific deficits were shown in the prefrontal cortex, which is probably the nodal point of the circuit, in the thalamus and in the hippocampus of schizophrenic patients (Lewis 2002).

The human dorso-lateral prefrontal cortex is a key structure of the cortex that mediates the temporal organisation of behaviour *via* mechanisms such as attention and working memory (Fuster 2000) so that its dysfunction appears to be a central feature in the pathophysiology of schizophrenia (Weinberger *et al.* 1986), particularly with reference to critical deficits on cognitive functions (Bunney and Bunney 2000). Thus, evidence showing reduced metabolic activity ("hypofrontality") in the prefrontal cortex of schizophrenic patients during performance of a cognitive task (Hazlett *et al.* 2004) proved a particularly robust finding. Moreover, the magnitude of the deficits in the prefrontal cortex was interestingly found to correlate with the presence and the severity of negative symptoms and cognitive deficits while the reduced metabolic activity within the thalamic mediodorsal nucleus and the temporal lobe correlated with negative and positive symptomatology, respectively (Hazlett *et al.* 2004). Such alterations in metabolic activity may explain poor performance of schizophrenic patients in working memory tasks requiring intact prefrontal functioning, such as the oculomotor delayed response task and the Wisconsin Card Sort and Tower of London Tests (Weinberger *et al.* 1986; Green *et al.* 1992; Park *et al.* 1999; Glahn *et al.* 2000).

In addition, further evidence of the implication of the prefrontal cortex in schizophrenia pathology is provided by neuropathological studies showing alterations in synaptic function in the prefrontal cortex (Lewis 1997; Selemon and Goldman-Rakic 1999; Mirnics *et al.* 2001). In relation to these findings, the diminished volume of prefrontal cortex grey matter observed in schizophrenic brain (McCarley *et al.* 1999) was suggested to be due to a decrease in the amount of axon terminals, distal dendrites and dendritic spines that represent the principal components of cortical synapses. Consistent with the interpretation that the dysfunction of the prefrontal cortex in schizophrenia involves a loss of synapses (Lewis *et al.* 1999; Selemon and Goldman-Rakic 1999; Woo and Crowell 2005), the expression of the synaptic vesicle protein synaptophysin was found to be reduced in the prefrontal cortex of schizophrenic patients (Glantz and Lewis 1997; Karson *et al.* 1999), similar to the expression of many genes encoding for proteins that regulate synaptic structure and function (Mirnics *et al.* 2000; Hakak *et al.* 2001; Mirnics *et al.* 2001; Vawter *et al.* 2001; Pongrac *et al.* 2002). Also consistent with this hypothesis

are *in vivo* proton spectroscopy studies showing reduced levels of N-acetyl aspartate (NAA), a putative marker of neuronal and/or axonal integrity, in the prefrontal cortex. Interestingly, the correlation observed between these changes and the degree of impaired activation in other brain regions during working memory tasks may suggest that neuronal abnormality within the prefrontal cortex could be involved in functional disturbances in the working memory network (Bertolino *et al.* 2000). Finally, anatomical studies of post-mortem prefrontal cortex from schizophrenic patients revealed a maldistribution of neurons originating from the cortical subplate which may result in altered connectivity of thalamocortical neurons (Bunney and Bunney 2000) and may interestingly be consistent with the neurodevelopmental hypothesis of schizophrenia (section 1.1.5.2).

Alterations in GABA transmission within the dorso-lateral prefrontal cortex strongly supported the implication of this brain region in schizophrenia, especially with regards to its cognitive disturbances. This long-suggested hypothesis, based on post-mortem observations of alterations in presynaptic cortical GABA markers, decreased activity of the glutamic acid decarboxylase (GAD) GABA-synthesis enzyme and decreases in GABA release and uptake within the DLPFC (Benes *et al.* 1991; Sherman *et al.* 1991; Benes *et al.* 1996; Simpson *et al.* 1998; Lewis *et al.* 1999; Blum and Mann 2002), was recently refined upon evidence of specific alterations of the chandelier subclass of GABA neurons and especially those located in the middle layer of the schizophrenic DLPFC (Lewis *et al.* 1999; Lewis *et al.* 2004).

Dysfunction of these cells, reflected by reductions in their parvalbumin (a neuronal calcium-binding protein commonly used to define this subclass of GABAergic interneurons specifically affected in the prefrontal cortex of schizophrenic patients) levels and in the number of axon terminals (Beasley and Reynolds 1997; Pierri *et al.* 1999; Lewis 2000), was found to impact on pyramidal neurons to which they project, leading to an upregulation of some GABA receptors at the pyramidal axon initial segment (Selemon *et al.* 1995; Volk *et al.* 2002). In fact, this reduction of GABA activity in the prefrontal cortex, which may result in a compensatory increase in GABA receptors at the axon initial segment of pyramidal neurons (Volk and Lewis 2002), was suggested to constitute the primary process impaired in the dorso-lateral prefrontal cortical circuit contributing to cognitive dysfunction in schizophrenia. The

pyramidal neuron axon initial segment, where chandelier neurons synapse, is indeed the site of generation of action potential whereby chandelier neurons exert powerful regulatory inhibitory control over pyramidal cell output (Lewis *et al.* 2004). Thus, the increase of GABA receptors at the axon initial segment may induce a major disruption in inhibitory neurotransmission on pyramidal neurons leading to elevated uncoordinated firing throughout the corticolimbothalamic circuit (Fuchs *et al.* 2001; Cunningham *et al.* 2004) and thereby compromising the synchrony of cortical activity that is required for working memory (Lewis *et al.* 2004).

Finally, evidence suggesting that disruption of the corticolimbothalamic circuit including the dorso-lateral prefrontal cortex may constitute a core feature of the pathophysiology of schizophrenia was further reinforced by the potential of this hypothesis to fit most major current theories of schizophrenia including the neurodevelopmental and dysconnectivity hypotheses.

1.1.5- Aetiological and pathophysiological hypotheses of schizophrenia

1.1.5.1- Synaptic plasticity and altered connectivity in schizophrenia

The hypothesis that schizophrenia does not result from focal brain abnormalities but from pathological connectivity within the brain is probably the oldest theory of schizophrenia. In fact, the term "schizophrenia" itself proposed by Bleuler in 1911 to reword the psychopathological concepts of psychosis postulated by Wernicke (1906), already suggested the "splitting" of different mental domains. This theme re-emerged recently in neurophysiologic and neuroimaging studies showing abnormal distributed activity and functional connectivity in schizophrenia (Volkow *et al.* 1988; Weinberger *et al.* 1992), leading to the hypothesis that schizophrenia may be regarded as a "disease of neuronal connectivity" (Andreasen 2000). Interestingly, this hypothesis may be consistent with evidence of alterations in neural circuitry, particularly in the cortex (section 1.1.4).

While the evidence suggesting brain disconnection in schizophrenia is thus accumulating, the underlying causes remain elusive (Stephan *et al.* 2006). Abnormal

functional coupling between brain areas may indeed be due to alterations of their anatomical connections or result from an impaired control of synaptic plasticity resulting in abnormal functional integration of neural systems (Friston and Frith 1995). Whichever is the primary impairment is still unknown (Krystal *et al.* 2003) and each of these mechanisms (which may not be exclusive but have a common cause) has in fact been supported by a number of observations.

The hypothesis, first proposed by Friston and Frith (1995), that dysconnectivity may result from an impaired "microconnectivity", *i.e.* the connectivity between adjacent cells and cortical microcircuits (Walterfang *et al.* 2006), leading to alterations in experience-dependent synaptic plasticity, is consistent with the NMDA receptor hypofunction hypothesis of schizophrenia (section 1.1.3) since NMDA receptors have long been shown to play a central role in the modulation of excitatory synaptic transmission and plasticity (Malenka and Nicoll 1993; Krystal *et al.* 2003). Following on this hypothesis, the mechanisms by which NMDA antagonists can induce schizophrenia-like psychotic symptoms and cognitive deficits may be mediated by alterations in the activity-dependent synaptic plasticity that underlies learning and memory.
In addition, further support for abnormal plasticity in schizophrenia has been provided by recent genome-wide linkage and allelic association studies which identified a number of NMDA receptor-dependent signalling and/or neurotransmitter-related genes (Stefansson *et al.* 2002; Martucci *et al.* 2003; Goldberg *et al.* 2006).

An alternative to this hypothesis may be the accumulating evidence of white matter changes and myelin-related dysfunction in schizophrenia, supporting the theory that schizophrenia may result from an anatomical impairment in "macroconnectivity", *i.e.* the connectivity between functionally related but anatomically disparate regions, in the schizophrenic brain (Davis *et al.* 2003; Walterfang *et al.* 2006). Myelination and factors that affects myelination, such as the function of oligodendroglia, are indeed critical processes that can profoundly affect neuronal connectivity, especially given the diffuse distribution of oligodendrocytes and the widespread distribution of brain regions implicated in schizophrenia (Davis *et al.* 2003).

Thus, the coincidence between the peak age of onset of schizophrenia (during adolescence or early adulthood) and the time when prefrontal and hippocampal connections achieve full myelination (Rapoport *et al.* 2005) strongly supports the hypothesis that altered myelination may play an important role in the disruption of brain connectivity in schizophrenia.

The range of evidence for white matter abnormalities in schizophrenia includes neuropathological studies showing a reduction in size and density of oligodendrocytes, gene expression studies showing the downregulation of a number of oligodendrocyte and myelination genes (potentially responsible for the reductions in oligodendrocyte density) and both volumetric and non-volumetric neuroimaging techniques identifying contradicting but accumulating white matter abnormalities (Walterfang *et al.* 2006). In particular, white matter volume was found to be reduced in the prefrontal cortex of schizophrenic patients correlating with the negative symptoms suggesting that white matter abnormalities may be involved in the disturbance in connectivity within and between the prefrontal cortex and other brain regions (Davis *et al.* 2003) and thereby may have a role in the disruption of the corticolimbothamic circuit in schizophrenia (section 1.1.4).

In conclusion, there is converging evidence suggesting alterations in connectivity in schizophrenia, and several hypotheses have been proposed to explain the putative basis of this dysconnectivity. These hypotheses may not be exclusive; for example, since glial cells are known to play an important role in neuronal migration and synaptic function (including NMDA regulation), abnormalities in white matter oligodendrocytes may be related to NMDA receptor function and altered synaptic plasticity in schizophrenia. Thus, oligodendrocyte dysfunction may lead to abnormalities such as reduced neuronal size, reduced levels of synaptic proteins and abnormalities in neurotransmission representing the basis of impaired synaptic plasticity and functional dysconnectivity in schizophrenia (Kubicki *et al.* 2005).

1.1.5.2- Neurodevelopmental hypothesis of schizophrenia

In recent years, schizophrenia has increasingly been regarded as a neurodevelopmental disorder (Murray and Lewis 1987; Weinberger 1987). Indeed, although different hypotheses have been proposed as to the timing and nature of

potential developmental disturbances, there is accumulating evidence suggesting that schizophrenia is the behavioural outcome of aberrations in neurodevelopmental processes beginning long before the onset of clinical symptoms and caused by a combination of environmental and genetic factors (Cardno *et al.* 1999; Singh *et al.* 2004). This theory is supported by epidemiological evidence showing increased obstetrical complications and childhood neuropsychological and motor deficits in individuals who subsequently develop schizophrenia, and neuroimaging studies showing ventricular enlargement and reductions in cortical volume at illness onset (Pantelis *et al.* 2003) and in people at higher genetic risk of the disorder (Lawrie *et al.* 1999). In addition, histological studies, though prone to methodological problems, have provided evidence for subtle cytoarchitectural abnormalities of putative developmental origin within the frontal lobes and temporo-limbic structures such as the hippocampus (Harrison 1999; Benitez-King *et al.* 2004).

Attempts to conceptualise the neurodevelopmental hypothesis of schizophrenia have mainly led to two models, one favouring an early "static" brain lesion model (Weinberger 1987; Gilmore *et al.* 1998) and one supporting a late disturbance in brain maturation (Feinberg 1982; Mathalon *et al.* 2003). The first model, supported by post-mortem studies showing abnormal patterns of neuronal migration, particularly during the development of the cerebral cortex (such as the disruption of cortical subplate activity; Bunney and Bunney 2000), suggests that the primary pathological event occurs during the pre- or peri-natal periods but does not become clearly evident until after puberty, when the affected networks become fully mature. Its functional consequences result then in disturbances in cortical connectivity and associative function (Weinberger 1987; Bunney and Bunney 2000). In contrast, the model favouring late developmental changes focuses on maturational events occurring during adolescence, such as the maturation of certain complex brain regions that are critical to meeting the challenges of adult life. This model is particularly supported by reproducible observations of reduced neuronal size and arborisation (Selemon and Goldman-Rakic 1999) suggesting that the pathophysiological processes involved in schizophrenia cannot be restricted to the pre- or peri-natal period (Rapoport *et al.* 2005). Therefore, although both models differ as to the supposed primary disturbances involved, they converge on a common view according to which late or ongoing developmental processes are critical to the

pathogenesis of schizophrenia (Lewis 1997). Interestingly, this is consistent with brain imaging studies showing a pattern of progressive changes both for early onset and chronic adult patients (Lewis and Levitt 2002; Rapoport *et al.* 2005).

In addition, evidence showing that the connectivity of the prefrontal cortex is substantially refined during adolescence suggests that these developmental changes are critical for the appearance of the clinical symptoms of schizophrenia (Lewis 1997). In the absence of classical degenerative changes, synaptic pruning, activity-dependent changes and myelination have been proposed as mechanisms whose alterations may underlie the development of schizophrenia during adolescence by leading to an abnormal reorganization or strengthening of cortical connections and therefore to alterations in connectivity and synaptic plasticity within critical cortical regions (Lewis and Levitt 2002; Benitez-King *et al.* 2004). Interestingly, this theory may not only provide further support to the dysconnectivity hypothesis of schizophrenia but a basis for the critical dysfunction of the dorso-lateral prefrontal cortex circuitry in schizophrenia (section 1.1.4).

The neurodevelopmental hypothesis of schizophrenia may therefore be consistent with most major theories of schizophrenia (sections 1.1.5) which explains why this model is probably the most largely accepted explanatory hypothesis about schizophrenia pathophysiology. Although the precise biological mechanisms underlying these putative changes remain unknown, the development of mature synaptic networks, which involves a dynamic interplay between genetic, environmental and stochastic factors, is likely to be the only process whose complexity may integrate the heterogeneity of schizophrenia and the complexity of its aetiology.

1.2- Approaches to gene identification in schizophrenia

Although epidemiological, pharmacological and neurobiological studies have definitely allowed considerable advances in the understanding of schizophrenia, they still only provide an incomplete picture of this highly complex disorder (Kirov *et al.* 2005). Moreover, because schizophrenia is a highly heritable but also multifactorial disorder, these studies cannot be considered exclusively and the most important advances in the comprehension of schizophrenia pathophysiology and aetiology will probably arise from their integration with genetic and genomic approaches to studying schizophrenia.

The identification of genes involved in the susceptibility and or in the pathology of schizophrenia is therefore of major importance. Nevertheless, in the context of the limited understanding of the aetiology and pathophysiology of schizophrenia, molecular genetic approaches have proven very difficult, especially because they not only share many of the problems encountered by other forms of schizophrenia research (including tissue availability, confounding factors such as medication) but also the problems inherent to complex disorders (including difficulties to reach statistical significance and/or to replicate findings). In addition, schizophrenia is such a heterogeneous disease in its presentation that it is very difficult to define a phenotype for human schizophrenia research, and to model it in any animal model.

Despite these issues, the more recent gene discovery studies have allowed the identification of a few well-supported candidate genes for schizophrenia whose interpretation may hold promising clues to understanding its neuropathology, giving insights into its causes and helping in the development of novel antipsychotic drug treatments.

1.2.1- The phenotype of schizophrenia

Besides being faced with the difficulty of the lack of a simple one-to-one relationship between genotype and phenotype (Owen *et al.* 2004), the identification of

susceptibility genes for schizophrenia is complicated by the limitations of the "phenotype" concept in psychiatric research in general (Sanders *et al.* 2004). Similar to most major psychiatric disorders, schizophrenia displays indeed an extensive heterogeneity of symptoms, course and outcome, and although aetiological subgroups have not been defined yet, it is likely that the disorder includes several different disease processes (Owen *et al.* 2005a). Moreover, although the use of explicit operational criteria based on structured and semi-structured interviews allows excellent diagnostic reliability, the inherited genetic vulnerabilities may not be specific to schizophrenia but result in a range of clinical phenotypes including a spectrum of disorders such as schizoaffective disorders and schizotypical personality disorder (Farmer *et al.* 1987; Kendler *et al.* 1995). In addition, independently of the extensive co-morbidity of psychiatric disorders, it is plausible that in a number of instances different psychiatric syndromes may share common aetiological genetic factors. In particular, the limits of the schizophrenia spectrum and its relationship to other psychiatric disorders, especially bipolar disorder (Kendler *et al.* 1998; Tienari *et al.* 2000) remain uncertain.

Nevertheless, the high heritability of the schizophrenia syndrome as defined by current diagnostic criteria was shown to make it theoretically suitable for molecular genetic analyses. Therefore in the absence of validated ways of defining sub-groups, most groups have chosen to search for genes using "schizophrenia" as a phenotype (Owen *et al.* 2004).

In parallel, as an attempt to "decompose" or "deconstruct" the syndrome of schizophrenia, endophenotypes, *i.e.* measurable variables which are intermediate between the genotype and the phenotype, have emerged as an important concept in the study of complex neuropsychiatric diseases (Gottesman and Gould 2003). These traits, which are not readily observable but require the use of special processes or instruments for their detection, are indeed assumed to have a closer relationship to the underlying disease genotype and thereby be less complex genetically (Cannon 2005). Several potential endophenotypes have been suggested for schizophrenia, including sensory-motor gating deficits such as deficits in pre-pulse inhibition (PPI), various event-related potentials (including the P50 event-related suppression) measured by electroencephalography (EEG), eye-tracking dysfunction and impairments in working memory. However, none of these has yet allowed the

identification of replicated susceptibility genes for schizophrenia (Owen *et al.* 2005a). In fact, the search for endophenotypes is not straightforward either because no *a priori* criterion can be used to decide if a particular element of schizophrenia reflects the effect of a single gene. Moreover, the assumption that the genetic architecture of endophenotypes is substantially simpler than that of schizophrenia itself is still under debate. Nevertheless, despite the lack of evidence of the relevance of endophenotypes as genetic "vulnerability markers", the current consensus is that endophenotype-based strategies may play an important and informative role to understand schizophrenia by revealing the genetically-mediated vulnerability factors that interact with non-genetic factors to produce this complex psychiatric disorder (Freedman *et al.* 1999; Braff 2002; Owen *et al.* 2005a).

1.2.2- Animal models of schizophrenia

The difficulty and the complexity of working with human schizophrenic samples have long stimulated the development of animal models of schizophrenia, despite the concern about the fact that less cognitively developed animals such as rodents may not faithfully reproduce what is perceived to be a disorder of the highest human brain functions (Marcotte *et al.* 2001). Nevertheless, in psychiatry like in any other area of medicine, the use of animal models is indeed a valuable tool for helping to understand the pathophysiological mechanisms because of the inherent greater simplicity of working and manipulating animals as opposed to human subjects; this includes the availability of tissue, the freedom in designing experiments and the possibility of controlling many more variables, of testing particular hypotheses etc (Wong and Van Tol 2003). Nevertheless, schizophrenia is such a complex disease, both in its aetiology and in its clinical heterogeneity and putative pathophysiology, that developing a valid model represents a major challenge.

Therefore, in order to avoid misunderstandings or potential excessive extrapolations, it is very important to specify first what the model is intended for and therefore what criteria it should meet when developing and evaluating an animal model (Geyer 2002b). Thus, at two extremes, "animal models" of schizophrenia may well refer to attempts to mimic the schizophrenia syndrome in its entirety or to develop a model for the study of antipsychotic drug treatments. Development of the former is

necessarily confronted with the impossibility to reproduce the heterogeneity of schizophrenia and the complexity of its clinical course while the latter, being validated by reference to the effects of known antipsychotics, may be limited in its potential to identify drugs with novel mechanisms of actions or that might better treat the symptoms not improved by current medications (Geyer 2002a).

In this context, the most common approach used to develop an animal model of schizophrenia has been to focus on specific signs, symptoms or aspects of schizophrenia that can be mimicked and observed reliably in animals, most often rodents (Gainetdinov *et al.* 2001). A number of models have thus been developed that mimic biological phenomena potentially associated with schizophrenia symptoms, including developmental changes, abnormalities in neurotransmission and genetic characteristics (Lipska and Weinberger 2000; van den Buuse *et al.* 2005). Interestingly, the combination of these animal models may further help to understand the neurobiology of schizophrenia, for example by providing insights into different mechanisms giving similar symptoms and thereby help to draw a more complete picture of the complex aetiology and pathophysiology of schizophrenia (Lipska and Weinberger 2000; Wong and Van Tol 2003).

Different types of animal models of schizophrenia include the lesion models and environmental models, which are based on various aetiological theories, pharmacological models based on abnormalities in neurotransmission and the more recent genetic models that directly investigate the role of particular genes in schizophrenia pathophysiology.

The PCP model, despite being a pharmacological model of schizophrenia, will be presented separately as it has emerged as one of the most promising models that may have the potential to bridge a number of hypotheses of schizophrenia.

1.2.2.1- Neurodevelopmental models of schizophrenia

A number of animal models have been developed to explore the neurodevelopmental theories of schizophrenia by testing different aspects of this hypothesis, including several aetiological hypotheses (lesion models), the impact of disrupted neurogenesis and the relevance of early stressful experience (environmental models).

In lesion models of schizophrenia, disruption of the neurodevelopment or the correct functioning of specific brain regions at presumably critical times of the development constitutes a highly controlled approach to explore the neurodevelopmental hypotheses of schizophrenia (Marcotte *et al.* 2001) and such models have been invaluable in helping to understand the pathophysiology and neurodevelopmental functions of various brain regions in relation with schizophrenia (Wong and Van Tol 2003).

Thus, a number of brain regions potentially involved in schizophrenia, including the prefrontal cortex, the thalamus and the hippocampus, have been the object of targeted lesions, typically involving excitotoxic agents which destroy neuronal tissue by stimulating excitatory glutamate release or acting as direct glutamate receptor agonists (Marcotte *et al.* 2001).

Adult lesion models were found to exhibit some neurochemical and behavioural deficits similar to schizophrenic patients, such as preattentional sensorimotor gating deficits (reflected by altered prepulse inhibition of startle (PPI) after apomorphine injection in adult rats with prefrontal cortex (PFC) lesions (Swerdlow *et al.* 1995)). However their validity as animal models of schizophrenia appears limited by the size and the nature of lesions realised in adult mature brain regions (Marcotte *et al.* 2001). Conversely, neonatal lesion models, which have the ability to demonstrate a delayed onset of symptoms corresponding to the clinical presentation of schizophrenia in humans, appear much more valid to test neurodevelopmental theories of schizophrenia. The neonatal ventral hippocampal (VH) lesion model may be of particular interest since it incorporates many features of schizophrenia, including enhanced dopaminergic neurotransmission, hyperactivity, altered prepulse inhibition of startle (PPI) and persisting deficits in spatial learning and memory (Lipska *et al.* 1992; Lipska *et al.* 1995; Chambers *et al.* 1996; Wong and Van Tol 2003), promising insights into dissecting and understanding the molecular circuitry involved in the pathophysiology of schizophrenia.

The development of models based on disrupted neurogenesis has been stimulated by evidence showing alterations in cortical cytoarchitecture potentially of developmental nature (Lipska and Weinberger 2000). One example of these models is *in utero* exposure to a mitotic toxin, methylazoxymethanol acetate (MAM) which destroys populations of rapidly dividing neurons. Very interestingly, animals having

undergone MAM manipulations exhibit morphological changes in a number of brain regions implicated in schizophrenia, demonstrate a variety of behavioural alterations such as locomotor hyperactivity, stereotypies, cognitive impairments and alterations in prepulse inhibition of startle (PPI), and show electrophysiological abnormalities that may underlie psychomotor disturbances in schizophrenia (Johnston *et al.* 1988; Lipska and Weinberger 2000). This model therefore demonstrates that perturbation in cortical development can produce some of the behavioural characteristics associated with schizophrenia.

Finally, environmental models have been developed to explore the long-lasting consequences of stressful experience for brain development and for shaping adult behavioural responses (Lipska and Weinberger 2000). Thus, environmental factors that have been proposed as aetiological factors for schizophrenia, including pre-weaning non-handling (Shalev *et al.* 1998), hypoxia (Schwarzkopf *et al.* 1992), isolation-rearing (Valzelli *et al.* 1977) or prenatal malnutrition (Morgane *et al.* 1993) were found to induce behaviours in rodents that may be compared to some features of schizophrenia in humans (Wong and Van Tol 2003). Finally, these models, which have also been used to provide evidence that there is an interaction between genetic predisposition and early life environmental factors, may also represent an interesting approach to study these interactions in the development of schizophrenia.

1.2.2.2- Pharmacological models

The development and use of pharmacological models based on the understanding or on hypotheses of neurotransmission alterations has been the major approach in preclinical schizophrenia research for many years (Marcotte *et al.* 2001). As an attempt to reproduce these alterations and particularly to explore the dopamine, serotonin (or serotonin-dopamine) and glutamate hypotheses of schizophrenia, a range of drugs were used, including dopamine agonists such as apomorphine and amphetamine (Hantraye 1998; Castner and Goldman-Rakic 1999), NMDA receptor antagonists such as phencyclidine (PCP), dizolcipine (MK-801) and ketamine (Noda *et al.* 1995; Reijmers *et al.* 1995; Sams-Dodd 1997; Sams-Dodd 1998), serotonin (5-hydroxytryptamine or 5-HT) 5-HT_{2A} agonists such as lysergic acid (LSD) and mescaline (Geyer 1998; Yamada *et al.* 1999) and neurotoxins (MAM, 6-

hydroxydopamine (6-OHDA), p-chlorophenylalanine; Johnston *et al.* 1988; Lillrank *et al.* 1995), the drugs being administered either to adult animals or targeted to specific periods of development (Marcotte *et al.* 2001). These models are limited by the current understanding of the fundamental basis of thought and cognition (Marcotte *et al.* 2001); however since most of them involve the administration of drugs that induce or exacerbate schizophrenic symptoms in humans, they generally have fairly good construct and predictive validities (Costall 1995).

The most studied pharmacological models of schizophrenia have long been based on the behavioural effects of psychostimulant drugs such as amphetamine (Lipska and Weinberger 2000). However, these dopamine-agonist models now appear to have limited validity since typical dopamine-induced stereotypies and locomotor hyperactivity are not thought to reflect the human condition of people with schizophrenia any more (Geyer 2002b). Nevertheless, dopamine agonists such as amphetamine and cocaine disrupt PPI in rodents, thereby mimicking one of the most robust behavioural deficits observed in schizophrenic patients, and their effects are interestingly reversed by antipsychotic drug treatment (Swerdlow and Geyer 1998). Thus, because of their very good predictive validity, dopamine-induced models are still largely used to test the efficacy of dopamine-antagonist treatments for schizophrenia (Geyer 2002b).

Interest in the serotonin-agonist models of schizophrenia was first driven by reports of the behavioural effects (namely hallucinations) induced by LSD, which appear to be mediated by its agonism properties at 5-HT_{2A} receptors (Glennon *et al.* 1984). These models were then proved to exhibit deficits in habituation, PPI and latent inhibition that may interestingly be relevant to both specific abnormalities exhibited by patients in the early stages of schizophrenia and to the effects of antipsychotics (Geyer 1998).

Development of glutamatergic models of schizophrenia, similar to the NMDA receptor hypofunction hypothesis of schizophrenia (section 1.1.3), was primarily based on the observation that non-competitive antagonists of NMDA glutamate receptors such as phencyclidine (PCP) and ketamine exacerbate symptoms in chronic stabilised schizophrenic patients and produce a behavioural syndrome in healthy humans that so closely resembles symptoms of schizophrenia that it is frequently misdiagnosed as acute schizophrenia (Fauman *et al.* 1976; Javitt and Zukin 1991;

Krystal *et al.* 1994). Thus, the remarkable similarity of PCP-induced behaviours with the diverse array of schizophrenia symptoms, including cognitive deficits such as impaired attention and working memory, has prompted the use of PCP in pharmacological models of schizophrenia in both basic and clinical studies. The PCP model, which was shown to exhibit excellent construct, face and predictive validity as a model of schizophrenia, is further detailed in section 1.2.2.4.

1.2.2.3- Genetic models

Neurodevelopmental and pharmacological models of schizophrenia have recently been complemented by the development of genetic models (Smithies 1993). Beside studies investigating strain differences -thereby the contribution of genetic factors- in schizophrenia-relevant behavioural traits such as PPI (Paylor and Crawley 1997; Swerdlow *et al.* 2000), genetically modified (transgenic and knock-out) animal models of schizophrenia were developed following two different approaches (Geyer 2002b).

In the initial approach, genetically modified mice were used to test specific hypotheses of relevance to schizophrenia, particularly hypotheses of alterations in neurotransmission. Thus, evidence of altered dopaminergic and glutamatergic transmission in the pathophysiology of schizophrenia led to the development of a number of mice models including dopamine D_2-, D_3- and D_4- (Ralph *et al.* 1999), glutamate $GRIN_1$- and $GRIN_2$- (Mohn *et al.* 1999; Miyamoto *et al.* 2001) and NMDA NR_1-receptor knock-out mice (Mohn *et al.* 1999).

In the second approach, genetically modified mice were used to investigate the individual role of candidate genes involved in schizophrenia pathophysiology and evaluate their potential contribution to the development of schizophrenia-associated behaviours or symptoms (Geyer 2002b). Among all generated mice, calcineurin, neuregulin, NR_1 and STOP (Stable Tubule Only Polypeptide) knock-out mice were found to exhibit a range of alterations or deficits that may be relevant to schizophrenia, confirming the potential involvement of these genes in schizophrenia aetiology or pathophysiology (Geyer 2002b).

All these models have their own advantages and limitations. Lesion and environmental models appear interesting tools to test new theories of the origin of

schizophrenia and its potential mechanisms; they have been particularly useful to confirm that disruption of neurodevelopment affects dopaminergic neurotransmission leading to schizophrenia-related behaviours. Pharmacological models are very valuable in preclinical research, particularly to screen novel potential antipsychotic treatments. Finally, genetic models of schizophrenia appear very promising since they may allow us not only to test the potential role of particular genes in the development of schizophrenia but also to investigate the interactions between genetic and environmental factors that are thought to be critical in schizophrenia pathogenesis.

1.2.2.4- The chronic PCP model of schizophrenia

Accumulating evidence over the last decades has suggested the PCP model as one of the more valid models of schizophrenia, both in its ability to mirror schizophrenia pathophysiology with impressive precision and also as a very valuable tool to screen for novel antipsychotic medications.

The synthetic drug phencyclidine (PCP) was first developed as a surgical anaesthetic but rapidly withdrawn because of its secondary effects including hallucinations, disordered speech, delirium, agitation and disoriented behaviour. However, observations of the ability of PCP to trigger such experiences exactly prompted its illicit use as a hallucinogen and at the same time attracted interest of neuroscientists, intrigued by the similarity between PCP-induced behaviours and the symptoms of people with schizophrenia (Murray 2002).

PCP is a non-competitive antagonist of NMDA glutamate receptors that, similar to dizolcipine (MK-801), acts by binding to a site within the pore of the open NMDA channel (hence the term "open channel" blocker). However, PCP also exerts inhibitory effects on other receptors such as nicotinic acetylcholine and voltage-dependent sodium and potassium ion channels (Vincent *et al.* 1983; Oswald *et al.* 1984; Ffrench-Mullen and Rogawski 1989) and sigma, dopamine D_2 and serotonin 5-HT_2 receptors (Kapur and Seeman 2002). Nevertheless, since these actions are much less potent than its action on NMDA receptors, the effects of PCP in the

central nervous system appear to be mostly mediated by its antagonism at NMDA receptors (Morris *et al.* 2005).

Initial observations of the effects of PCP in humans prompted the use of PCP to model schizophrenia. However, although chronic exposure to PCP appears more relevant to model psychosis, it is only recently that efforts have been made to find the treatment regimes in rodents that may be equivalent to those inducing psychogenic effects in humans (Morris *et al.* 2005). A number of studies thus used single acute administration of PCP. In rodents and monkeys, acute sub-anaesthetic doses of PCP were shown to induce an array of symptoms relevant to schizophrenia symptomatology, including amphetamine-like increased locomotor activity and enhanced stereotyped behaviours (Javitt and Zukin 1991) but also cognitive and sensorimotor gating deficits (disruption of PPI) and impaired social interactions (Steinpreis *et al.* 1994; Sams-Dodd 1995; Swerdlow and Geyer 1998; Yamada *et al.* 1999). Moreover, PCP and other NMDA receptor antagonists acutely increase extracellular levels of dopamine and glutamate in the prefrontal cortex and alter firing of dopaminergic and nucleus accumbens neurons (Verma and Moghaddam 1996; O'Donnell and Grace 1998).

In contrast, repeated exposure to PCP was reported to produce different electrophysiological and neurochemical effects from single injections (Matsuzaki and Dowling 1985; Jentsch *et al.* 1997a; Hori *et al.* 2000), inducing a sustained decrease in dopamine turnover within the prefrontal cortex accompanied by deficits in working memory and prefrontal cortex-dependent tasks in both rats and monkeys (Jentsch *et al.* 1997a; Jentsch *et al.* 1997b). However, even if this treatment regime of PCP was proposed to be a useful model for some aspects of schizophrenia (Jentsch *et al.* 1997a), the relatively high doses of PCP that it employs may not be appropriate, since they are known to produce a pattern of degeneration (Olney *et al.* 1989) which is not observed in post-mortem tissue from schizophrenic patients (Morris *et al.* 2005).

As a consequence, chronic low-dose regimes of PCP administration have recently emerged. Interestingly, these models have proved not only extremely valid in their ability to mimic the symptoms of schizophrenia in humans and to predict the effects of novel antipsychotic drug treatments, but also extremely valuable in their capacity

to fit with a number of hypotheses of schizophrenia and explain a range of evidence of schizophrenia pathophysiology.

Thus, chronic low-dose PCP administration in rats was found to induce decreases in metabolic activity within the prefrontal cortex, reticular nucleus of thalamus and auditory structures, key structures displaying similar changes in schizophrenia (Cochran *et al.* 2003). Interestingly, the altered metabolic activity in the prefrontal cortex ("hypofrontality") in the schizophrenic brain was shown to correlate with the presence and severity of negative symptoms and cognitive deficits while altered metabolic activities within the temporal lobe and thalamus correlate with positive symptomatology, suggesting that this dose regimen may have the ability to induce an array of symptoms that may excellently match the condition of people with schizophrenia (Cochran *et al.* 2003).

In addition, chronic administration of PCP using this treatment regime was found to produce a selective reduction in prefrontal cortex *parvalbumin* mRNA expression indicating selective alterations in the activity of the basket and chandelier population of GABAergic interneurons (Cochran *et al.* 2003), consistent with alterations observed in post-mortem brains from schizophrenic patients (Lewis *et al.* 1999; Ohnuma *et al.* 1999; Pierri *et al.* 1999). This effect of PCP was also similar to data showing reduced *parvalbumin* mRNA expression in hippocampal interneurons after ketamine administration to rats for five days (Keilhoff *et al.* 2004). Interestingly, evidence showing that parvalbumin-containing interneurons are fundamental in local circuits within the prefrontal cortex recruited in working memory (Rao *et al.* 1999) and that blockade of $GABA_A$ receptors also disrupts working memory (Sawaguchi *et al.* 1988) strongly suggests that the decrease in parvalbumin observed in both schizophrenia and after chronic intermittent PCP treatment may be important in mediating the cognitive deficits and negative symptoms associated with schizophrenia (Cochran *et al.* 2003).

Finally, chronic intermittent administration of low-dose PCP was shown to induce a significant decrease in N-acetylaspartate (NAA) and N-acetylaspartylglutamate (NAAG) concentrations in the rodent temporal cortex which closely reflects post-mortem findings reported in schizophrenia and indicative of neuronal dysfunction (Reynolds *et al.* 2005).

Thus, chronic administration of low-dose PCP in rats was found to produce a pattern of neurochemical and metabolic changes that mirror those observed in the brains of schizophrenic patients with impressive precision (Morris *et al.* 2005). Nevertheless, in addition to this excellent face validity as a model of schizophrenia, this model also appears a very valuable tool for screening novel antipsychotic drug treatments. The metabolic changes induced by chronic low-dose PCP in the temporal cortex and thalamus, but not in the prefrontal cortex, were indeed shown to be reversed by chronic antipsychotic treatment, which mirrors the clinical observations of the ability of existing anti-psychotic drugs to restore altered metabolic activity in the thalamus and temporal cortex, but not the prefrontal cortex, in schizophrenic patients (Cochran *et al.* 2003). In the light of the correlation between prefrontal metabolic hypofunction and negative symptoms and cognitive deficits of schizophrenia, these observations may therefore reflect the efficacy of current anti-psychotic drugs against the positive symptoms but not negative symptoms and cognitive deficits of schizophrenia (Cochran *et al.* 2003). Hence this model may represent an unprecedented tool to evaluate the atypicality of antipsychotic drugs. Consistent with this hypothesis, there is evidence that in normal human controls the psychomimetic effects of ketamine are not blocked by typical antipsychotics whereas they are significantly reduced by the prototypical atypical antipsychotic clozapine (Malhotra *et al.* 1997a). The differential effects of typical and atypical antipsychotic drugs in reversing PCP-induced deficits are probably due to the fact that PCP and related compounds do not mostly exert their effects by increasing dopamine neurotransmission, which distinguishes the PCP model from monoamine-based models (Geyer 2002b). In fact, although these models involved single administrations of PCP, the first non-monoaminergic ligands (including a glycine-site agonist and a metabotropic glutamate-receptor agonist) which entered clinical trials had interestingly been based on preclinical studies using PCP models (Moghaddam and Adams 1998; Javitt *et al.* 1999).

The chronic PCP model described by Cochran *et al.* (2003) appears to mirror the pattern of neurochemical and metabolic changes observed in schizophrenic patients remarkably (Cochran *et al.* 2003). Moreover, there is preliminary evidence that the low-dose treatment regime used in this study produces a selective deficit in the ability to shift attentional set from one perceptual dimension to another, which is in complete accordance with the executive deficits revealed in patients by the

Wisconsin Card Sorting Test and characteristic of schizophrenic pathology (Joyce *et al.* 2002). Thus, although this regime does not cause any overt behavioural effects, it appears to induce the types of cognitive deficits seen in schizophrenic patients (Morris *et al.* 2005) and may therefore, unlike the dopamine-based models, show very strong construct validity for studying the cognitive and attentional deficits in schizophrenia (Geyer 2002b). In addition, the capacity of this model to predict and test the effectiveness of atypical antipsychotic drugs makes it an invaluable tool in preclinical research and for screening of potential antipsychotic or cognitive-enhancing drugs.

The ability of the chronic low-dose PCP model to mirror particularly well the cognitive and attentional deficits of schizophrenia (Morris *et al.* 2005) also makes it a very valuable tool to study some of the less elucidated aspects of schizophrenia pathophysiology. In this work, this model was thus used to identify genes associated with schizophrenia and particularly with the negative and cognitive deficits, which are also the most poorly improved by current antipsychotic medications.

1.2.3- Methods of gene identification in schizophrenia

Despite the difficulty of defining a phenotype for schizophrenia research and that of modelling it in an animal model, a number of strategies have been employed to identify susceptibility genes for schizophrenia. These approaches include in particular the traditional genetic approaches such as cytogenetic studies, linkage and association studies using genetic polymorphisms, as well as the more recent genomic or "transcriptomic" approaches investigating gene expression profiles in animal models or in schizophrenic patients by comparison with non-affected people.

1.2.3.1- Cytogenetic abnormalities

The identification of chromosomal abnormalities in affected individuals has been widely used to locate susceptibility genes for schizophrenia (Blair *et al.* 2005). Cytogenetic abnormalities such as translocations and deletions can indeed be valuable to point out a gene or a region somehow affecting the susceptibility to

develop schizophrenia (by directly disrupting the function of a gene, by having a positional gene effect on gene expression or by showing linkage with another susceptibility variant (Bray and Owen 2001)). However, because of the high prevalence of schizophrenia and because of its complex genetic aetiology, one single report of a cytogenetic abnormality is not sufficient to conclude a causal relationship with schizophrenia; such a contribution needs to be "validated" somehow in a larger population or in another context. Thus, chromosomal abnormalities may only be considered of possible significance if they are found in greater frequencies in affected individuals (or, if the abnormality is rare, if there are multiple independent reports showing its coexistence with schizophrenia), if they colocalise with a region showing positive linkage or association or if they co-segregate with schizophrenia in affected families (MacIntyre *et al.* 2003). Nevertheless, this approach has yielded some intriguing findings. In particular, the identification of a t(1;11) balanced reciprocal translocation segregating with schizophrenia in a large Scottish family (St Clair *et al.* 1990) has allowed the identification of two genes, of which one (*Disrupted in Schizophrenia 1, DISC1*) may have a critical role in the development of psychosis (Porteous *et al.* 2006). Valuable insights were also provided by evidence showing higher rates of schizophrenia with people with Velo-cardial-facial syndrome (Murphy *et al.* 1999), a condition associated with deletions of chromosome 22q11, within which two genes, *catechol-O-methyltransferase* (*COMT*) and mito*chondrial enzyme praline deshydrogenase* (*PRODH*) are now well-supported candidate genes for schizophrenia (Bray and Owen 2001; Owen 2005).

1.2.3.2- Linkage studies and positional cloning

For most patients, the susceptibility genetic variations cannot be found using classic or molecular cytogenetic methods. Genetic linkage approaches are thus used to examine the segregation of schizophrenia with alleles of polymorphic genetic markers in families with several affected individuals (Bourgeron and Giros 2003). The principle of this approach is that markers that are close to a gene tend to be inherited with it, therefore co-segregation of the disorder with a particular marker allele is suggestive of genetic linkage to a pathogenic locus.

The availability of well-characterised markers spaced across all chromosomes has enabled genome-wide linkage studies that may have the potential to locate disease

genes without any prior knowledge of disease aetiology (Owen *et al.* 2004). However, because this approach is ideally suited to detect genes of large effect in genetically simple disorders, the results of linkage studies in schizophrenia have been disappointing. In particular, a number of linkage studies did not reach genome-wide significance, and attempts at replicating findings were often unsuccessful. This may be explained by the fact that small genetic effects probably act in combination therefore their stringent identification may require very large sample sizes, and by the use of marker maps of insufficient density to extract fully any genetic information (Suarez 1994).

Nevertheless, more than 20 genome-wide studies have now been reported and sample sizes have been increased, allowing some patterns to emerge, with some linkages reaching genome-wide significance on their own (according to the criteria set forth by Lander and Kruglyak, (Lander and Kruglyak 1995) and some others being supported by more than one study (Kirov *et al.* 2005; Owen *et al.* 2005a). Moreover, two meta-analyses of schizophrenia linkage data have been recently reported (Badner and Gershon 2002; Lewis *et al.* 2003) which, unsurprisingly owing to the different methods they employed, obtained overlapping but somewhat different results. Thus, the study of Badner and Gershon (2002) supported the existence of susceptibility genes on chromosomes 8p, 13q and 22q whereas that of Lewis *et al.* (Lewis *et al.* 2003) most strongly favoured 2q. All together, the 8p and 22q regions were the only regions that were supported by both meta-analyses whereas nine regions were only supported by one.

These linkage data therefore support the predictions made by Risch (1990), who was suggesting, on the basis of genetic epidemiological studies, that it is highly unlikely that there is a locus of major effect on schizophrenia susceptibility. Conversely, there is evidence implicating a number of regions, which is consistent with the existence of multiple susceptibility alleles of small or moderate effect rather than one single locus of major effect (Kirov *et al.* 2005). One way of removing any doubt would be to study larger samples (for example 800-1,000 families), however given the cost of sample collection, another approach may be to use existing samples but to extract as much information as possible, for example by using diagnostic information when it is sufficiently characterised (Evans and Cardon 2004). Such an approach may be particularly advantageous since this information may in fact correspond to

intermediate phenotypes, which, despite lacking evidence of simple "genetic architecture", have already proven very valuable to confirm and extend linkage findings in schizophrenia (Paunio *et al.* 2004).

1.2.3.3- Association studies

The limited power of linkage analysis to detect genes of moderate to small effect has stimulated the interest in the association design, which is a more powerful method of identifying such genes, especially in feasible sample sizes (Risch and Merikangas 1996). In this approach, the aim is to detect gene variants showing a different relative distribution in unrelated groups of affected and non-affected individuals (Bray and Owen 2001). Association studies can be used to evaluate the contribution of specific genetic polymorphisms within candidate genes, most often bi-allelic single nucleotide polymorphisms (SNPs) that may influence the functioning or the expression of the molecule. Thus, the choice of candidate genes gives an important flexibility to this approach by directly addressing the association of specific candidate genes, whether they are functional (*i.e.* genes encoding proteins implicated by an aetiological hypothesis) or positional candidates (*i.e.* genes mapping to regions implicated by previous linkage studies) (Owen *et al.* 2004). Because the robustness of linkage findings in schizophrenia is questioned, most studies have focused upon functional candidate genes.

However, despite the potential of the genetic association approach to identify genetic variants affecting susceptibility to schizophrenia, these studies are associated with a number of potential problems (Owen *et al.* 1997), making it necessary to be fairly cautious when considering their findings -at least until a consistent pattern of replication has emerged (Bray and Owen 2001). Most studies performed to date do not have sufficient power to detect reliably or confirm the presence of alleles of small effect and the combination of low power and low prior probability implies that a number of reported positive findings are more likely to be due to chance rather than reflect a genuine association (Owen *et al.* 2004). In addition, even in the best-designed studies (with larger samples etc), potential differences in the genetic structure of different populations (population stratification) may cast doubt on positive association findings, which also suggests that findings on samples collected

from different groups cannot probably be considered true replication experiments (Jurewicz *et al.* 2001).

Nevertheless, association studies have benefited greatly from the emergence of more systematic genomic approaches. Thus, it is now possible to screen a region (such as a linked region) for potential association and even to investigate simultaneously many thousands of markers (hence genes) using high-throughput microarray platforms. Moreover, the recent revelation of the first haplotype map of the human genome based on extensive SNP genotyping (2005) constitutes a major progress in genetic analysis of complex phenotypes such as schizophrenia. It should enable the consideration of both haplotypes (*i.e.* particular patterns of sequential SNPs on a single chromosome that are inherited as a block from a common ancestor) rather than individual SNPs and the selection of SNPs that capture the most information for a genomic region, two approaches that may be especially relevant to schizophrenia. Thus, haplotype-based analysis was shown to reduce the dimension of statistical tests for association and potentially improve their statistical power (Clark 2004). Moreover, defining allelic variants of genes associated with schizophrenia may constitute a useful tool in characterising the extent of allelic diversity of potential susceptibility genes (Hennah *et al.* 2004). In addition, selecting a reduced number of markers representing large genomic regions may not only reduce the cost of association studies but may also make it possible to optimise whole-genome association screens in particular (Kruglyak 2005). Although this approach is still controversial, mostly because of the lack of evidence of the relevance of haplotype-based SNP selection in populations outside those used for the HapMap project (Montpetit *et al.* 2006; Tenesa and Dunlop 2006), it has already proven highly valuable in increasing efficiency and power to detect rare causal alleles (at the cost of reduced power to detect common causal alleles) which is particularly relevant to the likeliest hypotheses on the genetic susceptibility to schizophrenia (de Bakker *et al.* 2005).

1.2.3.4- Gene expression studies

Another approach for identifying schizophrenia susceptibility genes has been provided by the development of expression studies that, as a complementary

approach to linkage and association studies, have the potential to identify not only the genetic component of the illness but any environmental or other causative factor and to reveal novel molecular mechanisms that contribute to disease. In particular, genome-wide expression studies can enable the identification of novel candidate genes that may not have been previously implicated in schizophrenia, on the basis of their dysregulation in tissues from people with the disorder.

Molecular differences between patients and controls have been reported in many post-mortem studies and a range of methods have been used to identify genes potentially over- or under-expressed in individuals with schizophrenia. Thus, methods such as *in situ* hybridisation, quantitative polymerase chain reaction (qPCR) or northern blotting have been used to examine the expression of individual genes (usually one at a time) with a high specificity and sensitivity that in a way counterbalances the disadvantage of these techniques of being time- and tissue-consuming. In addition, functional genomic methods such as subtraction by hybridisation (SBH), serial analysis of gene expression (SAGE), total gene expression analysis (TOGA), massively parallel signature sequencing (MPSS) have emerged for high-throughput screening of differentially expressed genes in diseased *versus* control tissues. Nevertheless, although each of these techniques offer certain advantages, they are usually more time-consuming and less reliable in detecting differences in gene expression than microarrays (Vawter *et al.* 2001).

1.2.3.4.1- Microarray studies

Microarrays have become standard exploratory tools in drug discovery as well as drug development and clinical trials (Brazeau 2004). The opportunity they provide to analyse thousands of genes at a time is particularly relevant and offers huge potential to psychiatric disorders presumably of oligogenic or polygenetic susceptibility such as schizophrenia. Two widely accepted types of microarrays are currently used for gene expression analysis: cDNA and oligonucleotide microarrays, whose major manufacturer is Affymetrix. Although both types of microarrays have strengths and weaknesses, the Affymetrix GeneChip® technology offers several advantages, including the presence of multiple oligonucleotide probes on the chip that interrogate different regions of the same gene, quantitative results, good nominal sensitivity and

the possibility of post-hoc comparison of data across many microarrays simultaneously.

Nevertheless, in addition to the complexities surrounding the phenotype issue in schizophrenia studies (section 1.2.1), these methods have a number of limitations including variations in expression as a result of polymorphism in genes unrelated to the disease, variation in expression due to the environment or other factors such as nutrition, variation in mRNA quality and variation in tissue preparation (Owen *et al.* 2004). Whereas most of these issues, except the complexity of analysing gene expression in the brain, may be overcome when investigating gene expression changes in animal tissue, they are particularly apparent and potentially important when microarray studies are applied to the analysis of human post-mortem tissue. Indeed, the limited availability of post-mortem brain material makes it unavoidable to include brains of high diversity with respect to age, race, post-mortem interval (critical for mRNA quality), medication history and other factors that greatly affect the results of gene expression studies (Mirnics and Pevsner 2004).

Whatever the species, the complexity of brain tissue must be considered when analysing and interpretating the outcome of microarray experiments. Thus, the immense phenotypic diversity of the brain may dilute out transcriptome changes in specific populations of brain cells, potentially pushing them back below microarray detection (Pongrac *et al.* 2002). Another issue that may explain the small magnitude of changes commonly detected in microarray studies on brain tissue is the limited plasticity of the mature brain and nervous system in terms of mRNA *de novo* expression, induction and repression (Mirnics and Pevsner 2004). Because of these issues, gene expression changes found with microarrays on brain tissue are often only modest and hard to separate from experimental noise, which has a major influence on the analysis of their results. Finally, the interpretation of microarray data is further complicated by the predominant abundance of mRNA transcripts in the cell soma while the proteins are most often localised to axonal projections or nerve terminals, so that it is possible for the levels of the expression of a transcript to be up- or down-regulated while the protein is significantly regulated in the opposite direction. Overall, the number of factors influencing gene expression changes in such microarrays makes it necessary to be extremely cautious when interpretating detected

fold changes that may not reflect functionally significant structural or chemical changes (Mirnics *et al.* 2001; Mirnics and Pevsner 2004).

Therefore, statistical analysis of microarray experiments appears essential to ensure the validity of the final data. A variety of methods have been used in the three main phases of microarray data analysis: background adjustment and data normalisation (data pre-processing), statistical analysis to determine the significance of the changes in expression levels of individual genes, and identification of gene expression patterns such as clustering, multidimensional scaling or pattern identification (Chuaqui *et al.* 2002). In particular, among the various algorithms which have been developed to improve the one provided by Affymetrix, Robust Multichip Average (RMA) (Irizarry *et al.* 2003) has emerged as a very rigorous method of data pre-processing (Bolstad *et al.* 2003). Significance Analysis of Microarrays (SAM) (Tusher *et al.* 2001) is considered as a very rigorous method of identifying differentially expressed genes by using an inferential statistical analysis. In particular, SAM software analysis tool may provide a reasonable balance between conservatism and tolerance by calculating a false discovery rate (FDR, *i.e.* the probability that a given gene identified as differentially expressed is a false positive) that can reduce false-negative (type II) error rates compared to multiple measurements corrections (Mirnics and Pevsner 2004). This approach may especially be useful for hypothesis testing in samples of reduced size (Jeffery *et al.* 2006).

1.2.3.4.2- Confirmation of microarray results

As there is currently no consensus about data analysis, confirmation of microarray results by an independent method appears essential to ensure their validity, particularly in brain disease microarray research where gene expression measurements can be affected by a large number of factors. There are two approaches to independent confirmation of microarray data: *in silico* analysis and laboratory-based analysis (Chuaqui *et al.* 2002). However, although the *in silico* method provides a great opportunity to validate data without further experimentation, this approach is inherently limited to the information available for comparison in the literature and in public or private expression databases, both in terms of quantity and quality. This approach is therefore not widely used at present but it is likely that it

will become more useful when standardised methods for reporting array data, such as the MIAME format (Minimal Information About a Microarray Experiment, (Brazma *et al.* 2001), are commonly applied (Chuaqui *et al.* 2002).

By providing an independent experimental verification of gene expression levels, laboratory-based validation of microarray data has become a standard in microarray research that has now become an official editorial policy in several journals (Rockett and Hellmann 2004). Although the methodology used may be different depending on the scientific question, two techniques have recently emerged as particularly relevant to confirm gene expression changes in brain tissue: quantitative real-time PCR (qRT-PCR) and *in situ* hybridisation.

Because once established, quantitative real-time PCR (qRT-PCR) is a rapid, relatively inexpensive and highly sensitive method for accurate quantification of specific mRNAs (Chuaqui *et al.* 2002), it is considered by many to be the technique of choice for validating gene expression changes identified in microarray experiments (Mimmack *et al.* 2004). Several investigators have thus shown a good qualitative correlation between microarray and qRT-PCR results (Rajeevan *et al.* 2001; Yuen *et al.* 2002a), but this correlation seems to be poorer for genes exhibiting fold changes less than 1.5 (*i.e.* $< \pm 50\%$ expression) compared to those with fold changes greater than 1.5 (*i.e.* $> \pm 50\%$ expression) (Dallas *et al.* 2005). In quantitative terms, since fold changes are related to many parameters (including probe length and properties, absolute abundance of transcript, data standardisation) it would be inappropriate to directly compare microarray and qRT-PCR fold changes. In all cases, statistical significance has therefore to be the parameter considered when analysing gene expression data.

As an anatomical verification strategy, *in situ* hybridisation provides another important method of confirmation of gene expression that is particularly valuable in the brain since it can be used at multiple levels of resolution to confirm the presence of relevant mRNAs in brain regions, their localisation to subnuclei or layers and potentially the classes of cells in which they are expressed (Bunney *et al.* 2003). Nevertheless, the limited sensitivity of this technique suggests that *in situ* hybridisation should be considered more a qualitative than a semi-quantitative method of measuring mRNA expression. Therefore its value lies more in its potential

to extend microarray results anatomically rather than to provide an infallible confirmation of detected gene expression changes.

In conclusion, although gene expression, particularly microarray studies, have to face challenges with studies of brain tissue, these approaches have proven very powerful for the analysis of human brain disorders, allowing the identification of dysregulated individual genes and the implication of abnormal patterns of gene expression in schizophrenia (Mirnics and Pevsner 2004). Because microarray technology can provide such valuable insight into schizophrenia, it has become a mainstream technology for analysing gene expression as a complementary approach to molecular genetic studies of schizophrenia.

Although the genes whose expression changes are deemed of importance from microarrays are not necessarily the same as the ones classified as of importance from clinical, *in situ*, molecular, SNP association, knock out and drug perturbation data, the combination of these different approaches has allowed the identification of a few strong candidate susceptibility genes for schizophrenia and the emergence of converging hypotheses about its pathophysiology.

1.2.4- Current gene expression studies in schizophrenia

Because of the central role that has been attributed to the human dorso-lateral prefrontal cortex in schizophrenia pathophysiology, particularly as part of the corticolimbothalamic circuit (section 1.1.4), a number of microarray studies have been performed to investigate the expression of genes within this region following the hypothesis that such differentially expressed genes may be implicated in particular in the cognitive and attentional deficits of schizophrenia. Despite the limited availability of post-mortem tissue from schizophrenic patients, data are thus accumulating that report abnormal patterns of gene expression in the schizophrenic dorso-lateral prefrontal cortex compared with the same brain region of control individuals. In addition, as a complementary approach to avoid the complexities of working with human post-mortem brain tissue, a number of groups have chosen to use animal models of schizophrenia, particularly pharmacological models (including

the PCP model, section 1.2.2.4), to explore differences in gene expression as a reflection of gene expression changes in human schizophrenic patients.
Results of these studies are various and difficult to compare because of the diversity of microarray platforms and statistical analysis methods used, and because of the lack of general consensus on their validation.

Nevertheless, microarray studies on post-mortem dorso-lateral prefrontal cortex from schizophrenic patients have identified a number of genes and pathways potentially involved in schizophrenia, including genes related to synaptic function and plasticity, GABAergic and glutamatergic transmission, myelination, lipid metabolism and genes encoding metabolic or mitochondrial functions, which most often provided further support to existing hypotheses of schizophrenia pathophysiology (section 1.1.5).
Middleton *et al.* (2002) identified a decrease in the prefrontal cortical expression of several genes involved in the regulation of brain metabolism (Middleton *et al.* 2002) which is consistent with the highly specific pattern of metabolic alterations found in the prefrontal cortex of schizophrenic patients (section 1.1.4). Moreover, the abnormality in presynaptic or synaptic function that has largely been suggested from microarray studies (Mirnics *et al.* 2000; Mirnics *et al.* 2001; Vawter *et al.* 2002) based on the downregulation of a number of transcripts in the schizophrenic prefrontal cortex has been suggested to represent the molecular basis of the clinically observed hypofrontality of schizophrenic patients (section 1.1.4)
In addition, a number of microarray studies of the dorso-lateral prefrontal cortex of people with schizophrenia have provided further support to the dopamine and glutamate hypotheses of schizophrenia (section 1.1.3). Microarray analysis of an elderly cohort of schizophrenic patients (Hakak *et al.* 2001) thus revealed the upregulation of several postsynaptic transduction pathways known to be regulated by dopamine while the observed downregulation of genes involved in glutamate neurotransmission (Mirnics *et al.* 2000) and of markers of inhibitory GABA neurotransmission may contribute to the cognitive deficits characteristic of schizophrenia (Hashimoto *et al.* 2003). Interestingly, the study by Hakak *et al.* (2001), similar to that performed by Tkachev *et al.* (Tkachev *et al.* 2003) also revealed the downregulation of several key oligodendrocyte-related and myelin-

related genes, providing further support to the altered myelination and hence potential dysconnectivity hypothesis of schizophrenia (section 1.1.5.1).

In addition, cluster analysis of transcriptional alterations in the dorso-lateral prefrontal cortex of schizophrenic patients showed alterations in the expression of genes related to energy metabolism and oxidative stress, suggesting that oxidative stress and the resulting cellular adaptations are linked to the schizophrenia disease process (Prabakaran *et al.* 2004).

Finally, as a sort of bridge between the different hypotheses of schizophrenia pathophysiology, Middleton *et al.* (2005) recently reported consistent alterations in the expression of 14-3-3 genes in the schizophrenic dorso-lateral prefrontal cortex (Middleton *et al.* 2005). Although it is difficult to define precisely the appropriate context in which to view 14-3-3 gene alterations in schizophrenia, the integral role of the 14-3-3 family of proteins in regulating many aspects of cellular function in the brain, including signal transduction, synaptic neurotransmission (by interaction with markers of GABA neurotransmission and/or RGS4), neurotransmitter metabolism and mitochondrial function, may indeed interestingly be consistent with most hypotheses of schizophrenia so that these proteins may be central in the pathophysiology of schizophrenia (Middleton *et al.* 2005).

As a complementary approach to microarray studies on post-mortem tissue from schizophrenic patients, a number of groups have chosen to evaluate the effects of experimental manipulations on gene expression levels in relevant animal models of schizophrenia (Marcotte *et al.* 2003). Thus, a few studies have been performed which explore gene expression changes induced by PCP treatment in rats and mice (Ito 2002; Toyooka *et al.* 2002; Kaiser *et al.* 2004; Ouchi *et al.* 2005). Among these studies, that of Ito (2002), which was performed on the rat cortex using SAGE and a DNA array approach, reported that phencyclidine induced changes in gene expression related to neuroplasticity, including alterations in transcription factors, cell proliferation, apoptosis, cell adhesion and synaptic events other than neurotransmission (Ito 2002). In a similar approach but using the more reliable GeneChip® microarray technology, Kaiser *et al.* (2004) identified and independently confirmed the differential expression in the rat prefrontal cortex of a number of genes related to diverse biological processes including stress, inflammatory response, growth and development, neural plasticity and signal transduction (Kaiser *et al.*

2004). Moreover, further analysis of these results in the context of schizophrenia revealed that the dysregulation of a number of these genes may be related to hypotheses of schizophrenia pathophysiology such as those suggesting disruption of thalamocortical circuitry, alterations in neurotransmission and neuromodulation, abnormal myelination and altered brain lipid metabolism in schizophrenia (section 1.1.5).

In conclusion, the recent advances in molecular genetics and functional genomics (gene expression) approaches have allowed the emergence of a few strong candidate susceptibility genes for schizophrenia including *catechol-O-methyl transferase* (*COMT*), *proline dehydrogenase* (*PRODH*), *dystrobrevin-binding protein 1* (*DTNBP1*), *neuregulin* 1 (*NRG1*), *D-amino acid oxydase* (*DAAO*), *Disrupted in Schizophrenia 1* (*DISC1*) (first identified by cytogenetics) and *regulator of G protein signalling 4* (*RGS4*), whose roles have been found to be consistent with different hypotheses of schizophrenia pathophysiology. Although the discrepancies between the results obtained using different approaches or using similar approaches but different statistical analysis methods may be enormous because of the inherent issues of investigating the aetiology and pathophysiology of a complex and heterogeneous brain disorder such as schizophrenia, their combination has recently proven to be very valuable in identifying novel candidate genes for schizophrenia (Vawter *et al.* 2006) and forming novel hypotheses of schizophrenia disease mechanisms. Nevertheless, these approaches will further benefit from the standardisation of statistical analysis and the way of reporting of microarray data (MIAME initiative) but also from improved phenotype definition, probably through the use of validated endophenotypes relevant to schizophrenia.

1.3- Genomics and bioinformatics

The availability of the human genome sequence and the completion of the draft sequence of other species including the rat have provided a fantastic opportunity for scientists to explore the molecular mechanisms of human diseases and to reveal all possible human drug targets (Duckworth and Sanseau 2002). The analysis of these very large data sources containing the complete genomic DNA sequence of many species will indeed improve our understanding of biological systems, but it also requires sophisticated bioinformatics tools, in keeping with bioinformatics playing a more and more significant role in modern biological research (Yu *et al.* 2004).

Since "bioinformatics" is a general term which refers to the application of computational and analytical methods to biological problems, it includes both a number of methods to search for genes, patterns and inherent structure in biological data, and the development of new methods for database access and queries (NCBI). The term "computational biology" is more frequently used to refer to the physical and mathematical simulation of biological processes.

1.3.1- The human and the rat genome projects and their implication in genomic research

After ten years of international effort to unravel the human genome sequence and with a first draft announced in 2000, the official completion of the Human Genome Project was announced in spring 2003 (Collins *et al.* 2003), allowing free access to an extremely high quality genome sequence deposited in public databases. One year later, another large collaborative effort, the Rat Genome Sequencing Project Consortium (RGSPC) succeeded in decoding the genome sequence of the rat (Gibbs *et al.* 2004), the most widely used animal for studying physiology, nutrition and metabolism and a very useful animal for modelling human psychiatric diseases (Abbott 2004).

From then on, with sequencing of the rat genome complete (the third one being the mouse genome sequence, released in December 2002; Waterston *et al.* 2002), three

mammalian genomes were available for parallel analysis, opening up new perspectives for comparative evolution surveys, biomedical research and drug discovery and development (Kola 2004). In particular, comparison of genome sequences of the species is particularly valuable to identify unpredicted exons and other regulatory elements (Yu *et al.* 2004), especially in species whose genome sequencing has not and will not receive labour-intensive annotation efforts (Wu *et al.* 2004). This may be the case for the rat, since it is very unlikely that the first draft of the rat genome sequence, which covered about 90% of the genome (Gibbs *et al.* 2004), will be followed and complemented by further sequencing effort. Alternative approaches may therefore be necessary to reveal the remainder of the rat sequence and more importantly yet unidentified genes.

In order to make access to human and other species genomes as easy as possible for ordinary biologists, and following the lead of the group at the University of California, Santa Cruz (Kent *et al.* 2002), the National Center for Biotechnology Information (NCBI), the European Bioinformatics Institute (EBI) and others released their own genome browsers and analysis tools, each having its own design concept which may be useful to answer specific questions. Nevertheless, the Ensembl project (Hubbard *et al.* 2002), a joint operation of the European Bioinformatics Institute (EBI) and Sanger Institute, represents one of the most interesting developments in the analysis of human genome sequence since it provides high quality annotation of genes based on evidence from known protein, cDNA and EST sequences (Curwen *et al.* 2004). Moreover, the Ensembl Project offers a particularly user-friendly browser that has unique capabilities such as protein-based queries.

1.3.2- Approaches to gene prediction

Among the number of approaches which have been used to decode and understand the language of biological sequences, two distinct analytical themes have emerged: similarity-based and *ab initio* techniques. In the first approach, pattern recognition techniques are used to detect similarity between sequences and thus infer related structures and functions; in the second, *ab initio* prediction methods are used to deduce either novel genes or 3-dimensional (3D) structure of proteins from DNA

sequences by applying statistical patterns (Mathe *et al.* 2002). However, both approaches are not exclusive but additional, and the most effective gene prediction results to date have been obtained by the simultaneous analysis of large DNA sequences using both exon prediction software and similarity searches (Jones *et al.* 2002). The question of validity still remains if the putative gene is only predicted by one of the two methods.

1.3.2.1- Introduction to pattern recognition methods

The underlying principle inherent to the majority of sequence-similarity-based gene or protein prediction methods is the combination of similarity information with signal sensors (*i.e.* short motifs such as splice sites, promoters, poly(A) sites or translation initiation codon sites). This information can be acquired by a variety of sequence comparisons including genomic DNA/protein, cDNA/protein, genomic DNA/cDNA and genomic DNA/genomic DNA. In all cases, the concepts of sequence identity, sequence similarity, homology and orthology apply to the comparison so that their definition is fundamental to the accuracy of the description of alignment results. Pairwise sequence identity is thus defined by the extent (percentage) to which two (nucleotide or amino acid) sequences are invariant while pairwise sequence similarity refers to the extent to which nucleotide or protein sequences are related. The extent of similarity between two sequences can be based on percent sequence identity and/or conservation.

Homology refers to similarity attributed to descent from a common ancestor while orthology refers to homology between sequences in different species that arose from a common ancestral gene during speciation but may or may not be responsible for a similar function (NCBI). Identification of orthologues is critical for reliable prediction of gene function in newly sequenced genomes.

Pairwise comparison is based on database search algorithms that align sequences by inserting gap characters to bring them into vertical register and give alignment scores by counting the matched character positions. Scoring penalties are employed to minimise the number and length of gaps and matrices are used to score both identical and similar residues.

Two general models are currently used that view alignments in rather different ways: the first considers similarity across the full extent of sequences (global alignment), the second focuses on regions of similarity on parts of the sequences only (local alignment). Neither model is more correct than the other, they simply reflect different biological perspectives. The rationale for local similarity searching is that functional sites are localised to relatively short regions, which are conserved irrespective of deletions or mutations in intervening parts of the sequence. Thus, a search for local similarity may produce more biologically meaningful and sensitive results than a search attempting to optimise alignment over the entire sequence lengths.

There are many publicly available pairwise comparison programs; much faster are those that look for local alignments such as FASTA and BLAST (Basic Local Alignment Search Tool). These programs concentrate on finding short identical marches, which may contribute to a total match. They use implementations which address the issues of execution speed, making them usable with any basic computer hardware.

1.3.2.2- Sequence-similarity-based gene finders

After the pioneered Procrustes (Gelfand *et al.* 1996), a number of software packages based on similarity searches have emerged. The principle of most of these programs is to combine similarity information with signal information obtained by signal sensors, which may predict the presence of a functional site such as a splice site, a promoter, a poly(A) tail, a start or a stop codon. Programs aligning the genomic DNA sequence against a cDNA database have proven quite reliable with regard to the identification of exons independently of their coding status, particularly when the genomic sequence is aligned against cDNA from the same or a closely related organism (Fukunishi *et al.* 1999). The comparison of homologous genomic sequences also helps in the identification of conserved exons and allows for the simultaneous prediction of genes on both sequences (Do and Choi 2006). Programs such as TWINSCAN (Flicek *et al.* 2003) have also been developed which exploit the sequence conservation between two genomes in order to predict genes. In TWINSCAN, the initial alignments conducted using standard tools such as BLASTN (nucleotide-nucleotide BLAST) or TBLASTX (translated query *vs* translated

database) are used to inform the prediction algorithm, which predicts which regions of a genome are transcribed into pre-messenger RNA, how they are spliced, and which portions of the spliced transcript are translated into protein (Korf *et al.* 2001; Flicek *et al.* 2003).

1.3.2.3- Ab initio gene-finders

The majority of *ab initio* gene prediction programs are based on a range of underlying statistical properties of the coding regions, and use a variety of different mathematical techniques to locate all the gene elements that occur in a genomic sequence, including possible partial gene structures at the border of a sequence (Mathe *et al.* 2002). They rely on two types of sequence information: content sensors and signal sensors, which refer to short sequence motifs (such as splice sites, branch points, polypyrimidine tracts, start and stop codons) present in almost all eukaryotic genes. A variety of algorithms can be applied to the modelling of gene structure, including Dynamic Programming (DP), Hidden Markov Models (HMM) and Neural Networks (NN). For example, among the number of programs available, GRAIL and GeneID both use a Dynamic Programming approach but combined with Neural Network and Markov Model methods respectively (Do and Choi 2006). HMM-based gene finders, which represent a DNA sequence as the output of an abstract process that progresses through a series of discrete states, have proven the most successful, GENSCAN (which uses a generalised HMM model) being probably one of the most popular gene prediction programs (Guigo *et al.* 2000).

Overall, similarity-based prediction programs appear to be able to detect only a limited number of genes (low sensitivity) due to the lack of known mRNAs, whereas *ab initio* gene finders which rely on intrinsic gene measures (including coding potentials and splice signals) may predict a number of false positive exons and genes. Therefore, programs have been developed that combine two or more gene prediction programs, of different or similar types to predict genes from genomic structures (Do and Choi 2006). These include the Exon Union-Intersection and Gene Intersection algorithms, which perform exon and gene structure prediction by taking the intersection of overlapping HMMGene and GENSCAN predictions (Rogic *et al.*

2002), and programs such as GenomeScan, an extension of GENSCAN that incorporates similarity with a protein retrieved by BLAST (Yeh *et al.* 2001).

Combinations of similarity-based and *ab initio* approaches have been determined to perform efficiently in terms of gene annotation in large-scale genomic projects (Fleischmann *et al.* 1995) so that the integration of different gene prediction methods is a very promising approach that will undoubtedly be much further developed in the future (Mathe *et al.* 2002). In addition, attempts to integrate gene prediction programs with some protein structure prediction (such as the ORNL Genome Analysis Pipeline that uses predicted gene structure as an input to perform motif and protein predictions) also appear very promising and are likely to benefit from further development as well.

1.3.3- Expressed Sequence Tags (ESTs), a powerful tool for gene discovery

In addition to genomic sequences, expressed sequence tags (ESTs) provide a very robust sequence resource that can be exploited for gene discovery, genome annotation and comparative genomics (Rudd 2003).

ESTs are small pieces of transcribed DNA sequence (usually 200 to 500 nucleotides long) that are produced by typically unedited, automatically processed, single-read sequencing of random gene transcripts that have been converted into cDNA and cloned. Libraries of cDNAs are routinely prepared that contain tens of thousands of clones, represent a variety of specific tissues types and a snapshot of gene expression at defined developmental stages and/or following various challenges (Zweiger and Scott 1997).

The concept of using cDNAs to discover new genes was first established in the early 1980s (Putney *et al.* 1983) but it was only in 1991 that the term EST was first used in relation to gene discovery and the human genome project (Adams *et al.* 1991). Since these first publications, >16 million ESTs have been sequenced from more than 500 distinctly annotated species, representing a wide taxonomic variety of fungi, plants and animals. These ESTs are listed in dbEST, the EST database developed by GenBank (Boguski *et al.* 1993).

EST sequencing initially favoured the 5' end of directionally cloned cDNAs because the 5' sequences are likely to contain more protein coding sequence than the 3' ends, which often contain significant untranslated regions (UTRs). It is currently the converse, the 3' end being most often preferred because it is likely to offer more unique sequence (in many cases, the UTR) and can be used to distinguish between gene paralogues, *i.e.* genes related by duplication within a genome that have evolved new functions (Rudd 2003). Because of their nature, ESTs are thus not the complete representation of the parental cDNA but rather constitute tags that may be used for fishing genes within databases. Moreover, the EST sequence collections are only as good as the libraries from which they were generated and a number of contaminations have been reported, including xenocontaminations from foreign genomes or contamination from structural or regulatory sequences. Nevertheless, because ESTs represent a copy of the expressed part of a genome that may contain at least partial representations of genes not otherwise available in other resources, they have proven a very powerful tool in the search for novel genes and novel drug targets (Duckworth and Sanseau 2002). Thus, some companies have based their drug discovery efforts on identifying potential new targets from EST collections; for example, Human Genome Sciences (Rockville, MD, USA) have been very active in this field (Haseltine 2001).

Different approaches are used to mine EST databases for the presence of genes and proteins of potential biological interest. These include EST clustering, EST contig assembly and the use of a range of tools to search the EST databases and find coding regions (Jongeneel 2000).

1.3.3.1- EST clustering

Because of the way EST sequences are generated, large-scale EST sequencing projects have unsurprisingly generated many more EST sequences than there are expressed genes. Thus, much of the information found in EST databases is redundant, with many genes being represented by more than one EST. Therefore, in order to establish a non-redundant catalogue of the transcripts expressed in any specific tissue at any specific time or in any specific condition, it is necessary to

cluster EST sequences into groups that are likely to derive from the same mRNA species (Jongeneel 2000).

The most enduring effort at EST clustering is the Unigene project of the National Center for Biotechnology Information (NCBI) (Schuler 1997). In addition to EST data, it includes mRNA derived from known genes and "virtual" mRNAs deduced from the annotation of genomic sequences selected from Genbank. These elements, which are each given an accession number, are classified into clusters, each cluster putting together ESTs, known RNAs and gene transcripts that are thought to represent the same (and a unique) gene. In addition, many useful annotations are added to each cluster, including similarities to known genes, chromosomal localisation, libraries of origin of the ESTs represented in the cluster and tissue specificity of expression. Therefore the identification of an EST as a member of a Unigene cluster greatly enhances the amount of information that can be gathered about the corresponding gene.

Among the attempts to identify unique genes represented by EST data (Bouck *et al.* 1999), another important resource has been provided by the Institute of Genome Research (TIGR) through the creation of unique gene indices of clustered and assembled ESTs (Adams *et al.* 1995). Unlike the Unigene approach, the TIGR Gene Indices use assembly logarithms, rather than clustering, to produce tentative consensus sequences that represent the underlying mRNA transcripts. This approach has resulted in the production of larger numbers of high-quality contigs, where one gene may be represented in multiple entries (Quackenbush *et al.* 2000).

1.3.3.2- EST contig assembly

Assembling of ESTs potentially derived from the same transcripts such as the members of Unigene clusters has become a highly valuable tool to identify new genes and a number of attempts have been performed to develop methods specifically suitable for dealing with the particularities of EST sequences. In a problem similar to the generation of contigs from shotgun sequences (as is performed in large-scale sequencing projects), these approaches all aim at deriving a "consensus" sequence from the clustered ESTs by eliminating redundancy and reducing the error rate while increasing the length of the deduced mRNA sequence (Jongeneel 2000). Thus, a number of sequence assemblers can be used to generate

consensus sequence of ESTs, the most popular being Phrap (Green 1999), the TIGR Assembler (Sutton *et al.* 1995) and CAP3 (Huang and Madan 1999). However, since all of these are primarily designed to assemble genomic sequences, they do not necessarily take into account the specificities of EST assembly, generating a number of incorrect results (Malde *et al.* 2005). For example, ESTs, which represent gene transcripts, do not contain the repetitive sequences that are found in large amounts in the intragenic parts of genomic sequences. In addition, EST assembly is complicated by features like alternative splicing, single-nucleotide polymorphisms (SNPs) and post-transcriptional modifications. All these differences between genome and EST assembly make assemblers designed for genome assembly less applicable to ESTs (Pertea *et al.* 2003). Nevertheless, despite the need for specific tools to assemble ESTs and reconstruct gene transcripts, recent effort to improve the state of the art in sequence assembly tend to concentrate more on the issues of genomic than of EST assembly (Malde *et al.* 2005).

Therefore, although most of these programs are quite convenient, they are also quite error-prone in assembling ESTs therefore manual assembly remains a method of choice in the analysis of clustered ESTs (Jongeneel 2000). Thus, although the method requires extensive input from the user, it provides a much more flexible approach that allows not only all the specificities of ESTs but also some features such as the overlap of genes in the genome (sense-antisense transcription) to be dealt with. Nevertheless, because most alignment editor tools have been designed for contig assembly, manual EST assembly requires the use of alignment algorithms that are not intended to generate consensus sequences, such as the general purpose multiple sequence alignment program ClustalW. This method of EST assembly therefore necessitates a thorough manual inspection of the alignments generated to find the best fit and deduce a consensus sequence from aligned EST sequences (Jongeneel 2000).

1.3.3.3- EST-based similarity searches

Despite their incomplete and inaccurate nature, ESTs represent a highly valuable resource to speed up gene discovery and characterise genes expressed in normal and diseased tissues. Thus, the number of databases recently created to bring together ESTs (such as the dbEST created by GenBank) provide a resource for rapid

identification of novel genes, including novel gene transcripts and novel gene orthologues and homologues. The most commonly used method to perform sequence similarities searches using ESTs is BLAST (Altschul *et al.* 1990), a very fast algorithm that has been popularised by the availability of a powerful cluster of servers at the NCBI database (Jongeneel 2000). Nevertheless, in addition to these nucleotide *versus* nucleotide similarity searches providing a starting point as to the potential similarity between an EST or a cluster of ESTs and cDNA or genomic databases, another important application in drug discovery is to find new coding sequences more distantly related to the query sequence. In this case, comparisons are based on amino acid sequences rather than nucleotides, the simplest method for searching EST databases being a tool called TBLASTN, which essentially performs a six-frame translation of the database and does a standard BLAST against this virtual protein database. Alternatively, coding sequences may be extracted from the ESTs first (as long as the sequences are long enough) and then searched against this database using a tool called TBLASTP (Jongeneel 2000).

Overall, even though ESTs are a precious tool for genome annotation, the overall quality of any individual sequence within all collections of ESTs remains one of the major problems that affects homology screening with genomic sequences and therefore complicates gene identification (Rudd 2003). Assigning ESTs to genes based on DNA sequence homology screens is very subjective since no perfect alignment can be obtained, making a manual guess often necessary to evaluate the potential relatedness between an EST and a particular gene or transcript.

Despite these limitations, ESTs are at the forefront of technological change by providing a high throughput means not only to identify gene transcripts but also to monitor complex gene expression patterns. This potential has soon been realised by microarray manufacturers, the first one, Affymetrix, having released EST-based GeneChip® arrays (such as the rat genome U34 B and C chips) for several years. EST-based technologies have been used to understand disease processes and may enable biology to move from single gene to multigene, or even more complex epigenetic, explanations of diseases (Zweiger and Scott 1997).

1.4- Aims of this work

Schizophrenia is such a complex disease that trying to elucidate its neuropathological mechanisms is a real challenge. Using a range of different approaches, researchers have been trying for decades to tackle the issues associated with such a multifactorial disorder, whose clinical heterogeneity is likely to be the reflection of different pathological mechanisms. Although despite this complexity, neurochemical, neuropathological and more recently genetic and genomic approaches have yielded very valuable insights into the pathophysiology of schizophrenia and seem to converge on a few hypotheses as to its aetiology and neuropathology. However, the question as to whether schizophrenia is a single disorder or a "spectrum" of disorders still remains unanswered, especially since its criteria-based diagnosis, despite being quite reliable, enables two people with very different and sometimes almost non overlapping symptoms to be diagnosed with the same disease. Unless people had chosen to develop and use animal models of schizophrenia to overcome this issue, this heterogeneity of schizophrenia thus largely explains the lack of consistence between studies, failures to replicate findings and difficulty to interpretate results, which have characterised most studies for decades. It also explains why it is so difficult to develop novel antipsychotics, and why it is now commonly admitted that no single compound will be able to capture and improve all the features of schizophrenia.

In this context, several approaches have emerged, some of them prioritising the use of more specific phenotypes or even the development of diagnostic tools (similar to genetic tests already available for some cancer treatments) to identify subgroups of patients and develop or select more appropriate treatments, some others focusing on the most critical needs to guide the development of novel compounds with regard to currently available antipsychotics. Importantly, this approach was recently recognised by the American Food and Drug Administration (FDA) (as part of an initiative with the National Institute of Mental Health, NIMH) as essential through the identification of cognitive symptoms as critically unmet targets against which to specifically develop neurocognitive drugs.

By enabling to specifically model specific aspects of schizophrenia, animal models may be particularly valuable to this aim, and in this context, the chronic intermittent PCP model developed in our laboratory (Cochran *et al.* 2003) following on the NMDA receptor hypofunction hypothesis of schizophrenia, appeared of very high potential. This model was indeed shown to mirror the negative and the cognitive symptoms of schizophrenia with impressive precision, so that it may be a very valuable tool for investigating the pathophysiological mechanisms specifically involved in these symptoms and potentially identifying novel drug targets with this regard. Likewise, the chronic PCP model may be very useful to identify novel genes which may be involved in the pathophysiology of schizophrenia and more specifically in the negative and cognitive symptoms of this disease.

In order to fully exploit the potential of the chronic PCP model to identify novel and uncharacterised genes associated with the cognitive deficits of schizophrenia, microarrays allowing the screening of not only genes but Expressed Sequenced Tags (ESTs), represent a very suitable method for measuring gene expression. In the context of a large program aiming at identifying such genes, a transcriptome analysis of prefrontal cortex (*i.e.* one of the regions most often pointed out as being altered in schizophrenia and a critical region for the integration of attentional and working memory processes) dissected from rats treated with PCP according to the regime of Cochran *et al.* (2003), was thus performed using the rat oligonucleotide RG-U34 GeneChips from Affymetrix. After a very rigorous statistical analysis, a few hundred probe sets representing genes and ESTs were identified as differentially expressed in the rat prefrontal cortex following chronic PCP treatment (Catherine Winchester). Because ESTs, due to their incomplete and inaccurate nature, required more extensive bioinformatics analyses to potentially be converted into genes first, the analysis of probe sets representing ESTs (corresponding to RG-U34 B and C probe sets) was conducted separately from probe sets representing genes (RG-U34 A probe sets).

The specific aims of this work were to:

1) analyse the ESTs represented by probe sets differentially expressed in the microarray analysis of the rat prefrontal cortex following chronic PCP treatment, in terms of correspondence to known or predicted rat, mouse and human genes.

2) select from the list of genes identified from the conversion of ESTs, a few candidates for further characterisation, and confirm independently their differential expression in the rat prefrontal cortex after chronic PCP treatment.

3) investigate the mechanisms by which each of the two candidate genes whose differential expression was confirmed, may be involved in the pathophysiology of schizophrenia and, depending on the gene, characterise them further as potential drug targets or investigate their potential association with schizophrenia in humans.

CHAPTER 2: MATERIALS AND METHODS

2.1- Materials

2.1.1- General chemicals

All chemicals were obtained from Sigma-Aldrich and were AnalaR grade with the exceptions of the items listed in table 2.1.

Supplier	Chemicals
Antec International	Zircon
Cambrex Biosciences	Gelstar® nucleic acid gel stain
National Diagnostics	EcoScint scintillation solution
Fisher scientific	Glycine, Sodium hydroxide and Tris base
Invitrogen	Agarose
Promega	G418
Upstate	Rat tail collagen I

Table 2.1. *Chemicals*

2.1.2- Plasticware

RNase- and DNase-free microcentrifuge and PCR tubes were obtained from Thermo Electron.

Tissue culture flasks, plates, petri dishes, falcon tubes and cell scrapers were all from Corning Incorporated. Sterile fine tip pastettes were from Alpha laboratories.

Filter tips (Rainin instruments) were used on Pipetman® P air-displacement pipettes (Gilson).

Oakridge centrifuge tubes were from Nalgene.

Syringes were from BD Biosciences and Minisart 0.2μM syringe filters were from Sartorius.

Scintillation vials were from Gordon Keeble Ltd.

2.1.3- Glassware

Glass tubes were from Fisher scientific and coverslips were from VWR.

2.1.4- Enzymes

Restriction enzymes as well as terminal deoxynucleotide transferase were obtained from Roche. T4 DNA ligase was from Promega, Superscript III™ reverse transcriptase was from Invitrogen, *KOD* Hot Start DNA polymerase was from Novagen and Advantage™ 2 polymerase mix was from BD Biosciences.

2.1.5- Immunochemicals

Immunochemicals were obtained from a variety of suppliers (table 2.2).

Supplier	Immunochemicals
Abcam	Anti-EDG2 antibody
Abgent	Anti-EDG2 antibody
Cell Signalling	Mouse anti-phospho SAPK/JNK antibody HRP-linked anti-mouse and anti-rabbit antibodies
Cytoskeleton Inc.	Anti-(pan)-actin antibody
Exalpha Biologicals Inc.	Anti-EDG2 antibody
Invitrogen	Alexa Fluor® 488 phalloidin Polyclonal anti-rabbit immunoglobulins (IgGs) conjugated with Alexa Fluor® 488 Polyclonal anti-mouse immunoglobulins (IgGs) conjugated with Alexa Fluor® 596
Santa Cruz Biotechnology	HRP-linked anti-actin antibody
Sigma-Aldrich	Anti-EDG2 antibody Monoclonal anti-FLAG® M2 antibody HRP-linked anti-FLAG® M2 antibody
Upstate	Anti-EDG2 antibody

Table 2.2. *Immunochemicals*

2.1.6- Radioisotopes

Adenosine 5`-(α-thio)triphosphate, [35S] [α-^{35}S]-d-ATP (specific activity 1250Ci (46.2TBq)/mmol) was obtained from Perkin-Elmer, formerly NEN Life Sciences.

Guanosine 5'-[γ-35S]thiotriphosphate, triethylammonium salt (specific activity >1000Ci (37TBq)/mmol) was obtained from GE Healthcare, formerly Amersham Biosciences.

2.1.7- Oligonucleotides

Oligo-dT and random hexamers primers for cDNA synthesis were from Invitrogen.

Desalted oligonucleotides for PCR, RT-PCR and qRT-PCR were obtained from Sigma-Genosys. The oligonucleotides, their sequences and use are listed in table 2.3.
5'FAM/3'TAMRA-labelled oligonucleotide probes were obtained from MWG Biotech.
HPLC-pure oligonucleotides for *in situ* hybridisation were obtained from Thermo Electron.

Oligo-nucleotide	Nucleotide sequence 5'-3'	Purity	Modifi-cation	Use
B2m - F	CGTGATCTTTCTGGTGCTTG TC	Desalted		Primer for rat *B2m* qRT-PCR
B2m - R	GGCGAGAGTACACTTGAAT TTGG	Desalted		Primer for rat *B2m* qRT-PCR
B2m - p	CCGTCGTGCTTGCCATTCA GAAAAC	high purity salt free	5'FAM–3'TAMRA	Probe for rat *B2m* qRT-PCR
EST - F	TCATGAAGATTTAGCTGAG ATTTTGTG	Desalted		Primer for rat EST AI072720 qRT-PCR + RT-PCRs on EST + RACE PCR
EST - R	CAATCCCCATCAGGTATCA GGTA	Desalted		Primer for rat EST AI072720 qRT-PCR + RT-PCRs on EST + RACE PCR
EST - p	CAGAATCCTTCCCACTCCT GAACCTTGAA	high purity salt free	5'FAM–3'TAMRA	Probe for rat EST AI072720 qRT-PCR
Edg2 - F	GGTCAGGAGGATGTCTGTG AGAA	Desalted		Primer for rat *Edg2* qRT-PCR
Edg2 – R	CACGTCACATGCCTACCCT CTA	Desalted		Primer for rat *Edg2* qRT-PCR
Edg2 - P	CCCAGAATGCCACTCTCTG CAGGACTT	high purity salt free	5'FAM – 3'TAMRA	Probe for rat *Edg2* qRT-PCR
hGAPDH - F	CCCATGTTCGTCATGGGTG T	Desalted		Primer for human *GAPDH* qRT-PCR
hGAPDH - R	TGGTCATGAGTCCTTCCAC GATA	Desalted		Primer for human *GAPDH* qRT-PCR
hGAPDH - p	CTGCACCACCAACTGCTTA GCACCC	high purity salt free	5'FAM – 3'TAMRA	Probe for human *GAPDH* qRT-PCR
hEST – F	CATGGATGACACAATGTTT TTGC	Desalted		Primer for human EST qRT-PCR
hEST – R	CTTTCGTGTAAGATGGCGG TATAG	Desalted		Primer for human EST qRT-PCR
hEST - p	ACCCTGAAACTATTCTTTG GCATTGGTGTCC		5'FAM – 3'TAMRA	Probe for human EST qRT-PCR
hEDG2 – F	GGATGTCAGTGCACGGTTA GTG	Desalted		Primer for human *EDG2* qRT-PCR
hEDG2 – R	CTAATCGGGTCCCCACATT CT	Desalted		Primer for human *EDG2* qRT-PCR
hEDG2 – p	ACTACAGGTACTTTACAAG TCTGCCCTCTGCTCAA	high purity salt free	5'FAM – 3'TAMRA	Probe for human *EDG2* qRT-PCR
EST – RACE - R	AATCCCCATCAGGTATCAG GTACCAGAA	Desalted		Primer for 5'RACE on rat EST AI072750
Q9ULN1-1st-F	ACCTGAGAATGGCCAACAG C	Desalted		Primer for rat *RGD1308367* exon 1
Q9ULN – 2nd - F	CTTTGTTGTTCACCAGGCC AT	Desalted		Primer for rat *RGD1308367* exon 2
Q9ULN1 – 3rd - F	GAGAAGATGAGAGCGCAG CC	Desalted		Primer for rat *RGD1308367* exon 3
EST – 2as	GACAAGGAGGTTCTTTATT TAACAGCAGGTAGGTAGGT TGATAAT	HPLC		Probe for EST AI072720 *in situ* hybridisation
Edg2 – 3as	GACACAAGCCACACCCTGC CTTTCCTCCTCCCCAGAATG CCACTC	HPLC		Probe for *Edg2 in situ* hybridisation
Q9ULN1 – 2as	TCAGTTGTCTTCCTGGTTTG ACTGCTAGGCTGTTGGCCA TTCTCA	HPLC		Probe for *RGD1308367 in situ* hybridisation

Table 2.3. *Oligonucleotides* (F = forward primer and R= reverse primer)

2.1.8- Gene expression and SNP genotyping assays

Taqman® gene expression assays were obtained from Applied Biosystems.
Each gene expression assay consists of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers formulated into a single tube. Every assay is optimised to run under universal thermal cycling conditions and all assays have the same amplification efficiency.
Rat *RGD1308367* assay was a custom assay while human *KIAA1189* and *EDG2* assays were pre-designed assays.

SNP genotyping (Allelic Discrimination) assays were also obtained from Applied Biosystems. Each of these assays consists of a set of two PCR primers and two 5'-labeled Taqman® MGB probes (one labelled with FAM™ dye, the other one labelled with VIC™ dye) which selectively anneal to each of the alleles. 5' nuclease activity of Taq DNA polymerase allows discrimination between alleles by cleaving the 5' allele-specific dye label.
The three SNPs tested for association with schizophrenia were all pre-designed assays.

2.1.9- Drugs and solutions

Solutions and media were sterilised by autoclaving at 121°C for 15 minutes or by filtration through a sterile 0.2µm pore filter (Sartorius).

Lysophosphatidic acid (LPA):
Stock solution: 1mM in PBS/0.1% BSA (stored at -20°C)
Working solution: 0.1nM to 100µM

1 kb+ ladder:
0.1µg/µl 1Kb+ DNA ladder (Invitrogen) in 2X Blue Juice™ (gel loading buffer, Invitrogen)

10X TBE:
0.9M Tris, 0.6M H_3BO_3, 20mM EDTA (pH 8.0)

DL-Dithiothreitol (DTT):

Stock solution 1M in H_20 (stored at -20°C)

Guanosine diphosphate (GDP):

Stock solution 10 μM (stored at -20°C)

RIPA buffer:

50 mM Tris-HCl (pH 7.4), 1% (v/v) NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1% (v/v) protease inhibitor cocktail (final concentrations 1.04mM AEBSF, 0.8μM aprotinin, 40μM bestatin hydrochloride, 140M E-64, 20μM leupeptin hemisulfate and 15μM pepstatin A)

Buffer I for membrane preparation:

20mM Hepes, 1mM EDTA, 50mM NaCl (pH 7.5)

Buffer II for membrane preparation:

50mM Hepes, 2mM EDTA, 100mM NaCl, 1mM $MgCl_2$

GTP-binding buffer:

50mM Hepes, 100mM NaCl, 10mM $MgCl_2$ (pH 7.5)

3X SDS loading buffer:

187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% (v/v) glycerol, 150 mM DTT, 0.03% (w/v) bromophenol blue

5X SDS loading buffer:

312.5 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 50% (v/v) glycerol, 250 mM DTT, 0.05% (w/v) bromophenol blue

10X Running buffer:

0.25 M Tris, 1.9 M glycine, 1% (w/v) SDS

1X TBS:

0.2M Tris, 1.4M NaCl (pH 7.6)

10X Transfer buffer:

0.2M Tris, 1.5M glycine

Working solution: 100mL 10X transfer buffer, 200ml methanol and 700ml H_2O

Stripping buffer:

62.5mM Tris-HCl (pH 6.7), 2% (w/v) SDS, 100mM beta-mercaptoethanol

2.1.10- Media and antibiotics

2.1.10.1- Bacterial culture

Luria-Bertani (LB) medium and agar were made from Luria-Bertani broth and agar tablets (Sigma-Aldrich).

Ampicillin: stock solution 100mg/ml in H_20, working solution: 100µg/ml

2.1.10.2- Mammalian cell culture

Foetal bovine serum, penicillin-streptomycin solution (10000 units/ml penicillin and 10000 µg/ml of streptomycin), Ham F12 medium, Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine, 4500mg/L D-glucose and 110mg/L sodium pyruvate and Dulbecco's Modified Eagle Medium (D-MEM) with GlutaMAX™, 4500mg/L D-glucose and without sodium pyruvate were all from Invitrogen.

2.1.11- Kits

A number of kits were used for many different purposes. They are listed in table 2.4:

Supplier	Kit
Qiagen	RNeasy Mini kit
Qiagen	QIAquick Gel extraction kit
Qiagen	QIAquick PCR purification kit
Qiagen	Plasmid DNA mini and midi kits
Qiagen	QIAquick PCR purification kit
Qiagen	QIAquick Nucleotide removal kit
FLAG® tagged protein immuno-precipitation kit	Sigma-Aldrich
Pierce Biotechnology	SuperSignal West Femto Maximum Sensitivity Substrate
Amersham Biosciences	ECL+ (enhanced chemiluminescence) western blotting analysis system
Neurite outgrowth quantification kit	Chemicon

Table 2.4. *Kits*

2.1.12- Membranes and paper

PVDF membranes and filter paper for western blotting were obtained from Invitrogen. Saran was from Fischer Scientific.

2.1.13- Photography and autoradiography

Biomax™ MR X-ray films for *in situ* hybridisation (Kodak) and Hyperfilm™ ECL™ films for western blottings (GE Healthcare, formerly Amersham Life science) were developed using an automatic developer (Hyperprocessor, GE Healthcare, formerly Amersham Life science).

Agarose gels, western blotting and *in situ* films were analysed and photographed using a computer-based system (MCID5) either connected to a UV transilluminator (for agarose gels) or to a CCD camera (for films).

2.2- Experimental materials

2.2.1- RNA and DNA sources

Rat brain total RNA was obtained from Ambion.

Total RNA or first strand cDNA used for the rat qRT-PCRs were provided by Catherine Winchester (Strathclyde University, Glasgow). Total RNA had been extracted using the RNeasy Mini Kit (Qiagen) from the prefrontal cortex of rats treated with PCP or vehicle (n=6) according to YRING chronic PCP model treatment (*i.p.* injections of 2.58 $mg.kg^{-1}$ PCP once daily on days 1–5, 8, 10, 12, 15, 17, 19, 22, 24, 26 (Cochran *et al.* 2003). There were two collections of total RNA, one used primarily for a microarray study investigating gene expression changes in the YRING chronic PCP model (Catherine Winchester), the other one used of qRT-PCR. The quality of all samples had been confirmed by checking the electrophoretic pattern of the RNA using the Agilent 2100 Bioanalyzer (Sir Henry Wellcome Functional Genomics Facility, University of Glasgow).
First strand cDNA was synthesised from these total RNA samples (section 2.4.3).

The cDNAs used for the human qRT-PCR analysis were provided by Hiromitsu Ozeki (Mitsubishi Pharmaceuticals, Japan). They originated from brain tissue obtained from the Scottish Biomedical Foundation from different collections of schizophrenic patients and controls (namely Harvard, Brain-Net and UCLA). The purpose of this acquisition had been the realisation of a microarray to investigate gene expression in the human dorso-lateral prefrontal cortex of schizophrenic patients and this is what the tissue was used for first (Hiromitsu Ozeki).
Total RNA was extracted from post-mortem prefrontal cortex (Brodmann area 10) of schizophrenic patients and controls using the Qiagen lipid Midi kit (Hiromitsu Ozeki). Sample quality was confirmed using the Agilent 2100 Bioanalyzer (Sir Henry Wellcome Functional Genomics Facility, University of Glasgow). To enable a more detailed analysis, the samples were matched by pairs in order to take into account gender, approximate correlation of age and post-mortem delay, and if possible, cause of death (Hiromitsu Ozeki).
Table 2.5 shows known details of the samples.

First strand cDNA was synthesised using the protocol described in section 2.4.3 (Hiromitsu Ozeki).

source	Donor	source ID	gender	pm delay	Age	cause of death
brain collection	*control or schizophrenic*			*hours*	*Years*	
Harvard	control	B4810	F	17	62	not given
	control	B5245	M	16	58	not given
	control	B5270	M	23	52	not given
	control	B5287	M	25	66	not given
	schizophrenic	B4942	F	14	61	not given
	schizophrenic	B5047	M	23	63	not given
	schizophrenic	B5103	F	11	61	not given
	schizophrenic	B5115	M	25	49	not given
Brain-Net	schizophrenic	BN007/01	M	16	32	suicide
	control	HH008/01	M	10	61	heart attack
UCLA	control	482	M	26	24	homicide
	control	2546	M	20.5	57	non-hodgkin's lymphoma
	control	2556	M	27	74	myocardial infarction
	control	3236	M	16.5	68	laryngeal cancer
	schizophrenic	707	M	41	28	suicide
	schizophrenic	2211	M	17.5	73	coronary artery disease
	schizophrenic	2258	M	15.5	72	myocardial infarction
	schizophrenic	2586	M	19.5	58	adenocarcinoma

Table 2.5. *Information on human post-mortem prefrontal cortex samples*
F=female, M=male

2.2.2- Genomic DNA

Genomic DNA (gDNA) samples were obtained from Professor H.M. Gurling (Academic Department of Psychiatry, University College London Medical School, UK). These samples consisted of 300 control and 300 schizophrenic gDNA samples, distributed at a concentration of 20ng gDNA/µl (150µl per well in four duplicate 96-well microtitre plates). Blood had been collected from individuals of Anglo-Saxon parentage residing within the Greater London (UK) area.

Original samples were diluted 10-fold in stock dilution plates mixing controls and schizophrenic samples and including negative water controls to obtain a concentration of 2ng/μl for the allelic discrimination assays (section 2.4.8).

2.2.3- *EDG2* and *KIAA1189* cDNAs

pBluescript-*EDG2* and pBluescript-*KIAA1189* cDNAs clones were obtained from OpenBiosystems. Human verified full-length cDNAs were isolated from bacterial cultures of *E. coli* in LB broth with growth indicator, 8% (v/v) glycerol and selective antibiotic.

2.2.4- Bacteria and mammalian cells

Subcloning efficiency™ DH5α™ competent cells and one shot TOP10 competent *Escherichia coli* were obtained from Invitrogen.
The strains and their genotypes are detailed in Table 2.6.

Bacterial strain	Genotype
Subcloning efficiency DH5α™	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻
One shot® TOP10 competent cells	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Table 2.6. *Bacterial strains*

Human SK-N-SH and rat C6 glioma and PC12 cells were obtained from the European Collection of Cell Cultures (ECACC).

2.2.5- Vectors

Vectors were used for subcloning and cloning *EDG2* and *KIAA1189*: pcDNA3.1(+) (Invitrogen), pcDNA3.1-FLAG and pMSF1 (gifts from Mitsubishi Pharma Corporation, Japan) and pcDNA3.1/NT-GFP (Invitrogen) was used to visualise transfected cells. Their structures are shown in figures 2.1-4.

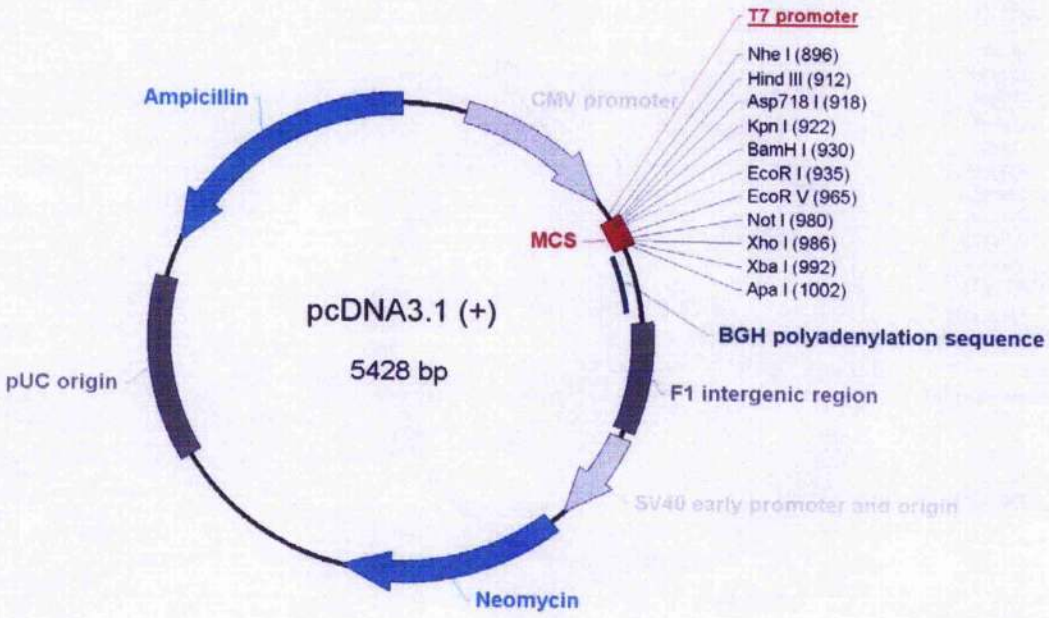


Figure 2.1. *Structure of pcDNA3.1(+) expression vector*

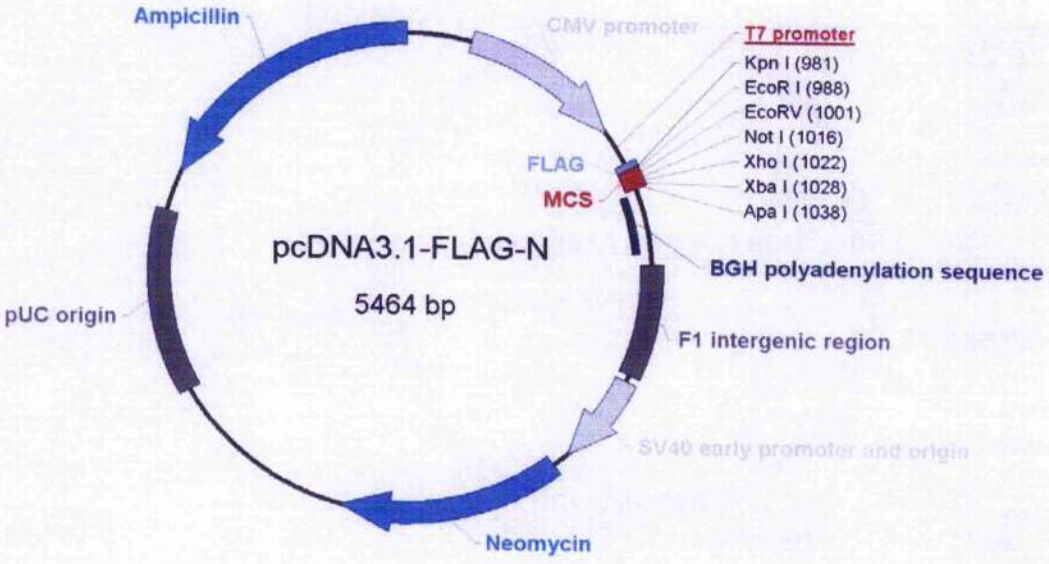


Figure 2.2. *Structure of pcDNA3.1-FLAG expression vector*

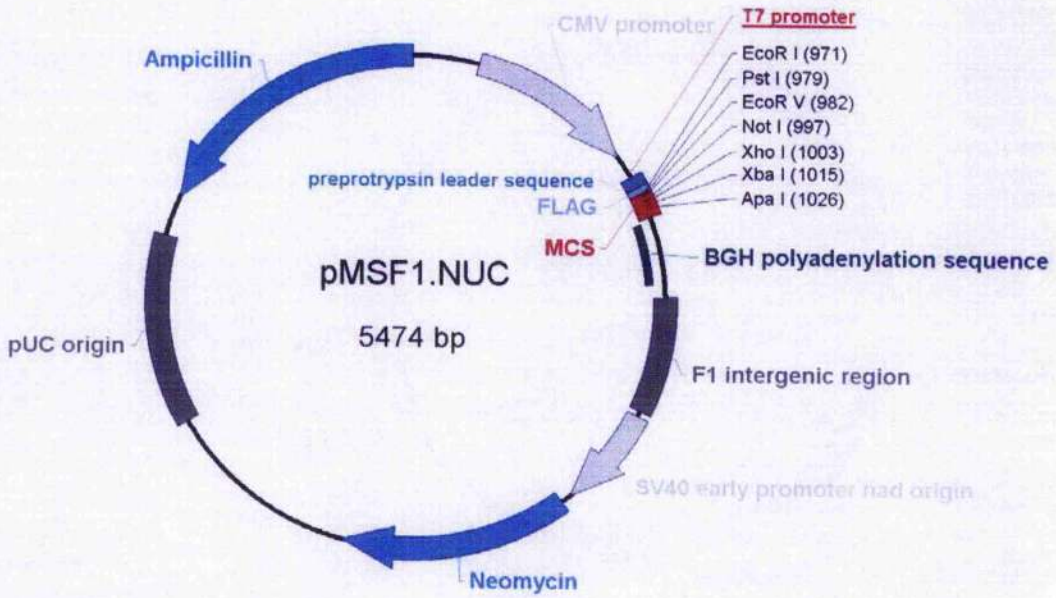


Figure 2.3. *Structure of pMSF1 expression vector*

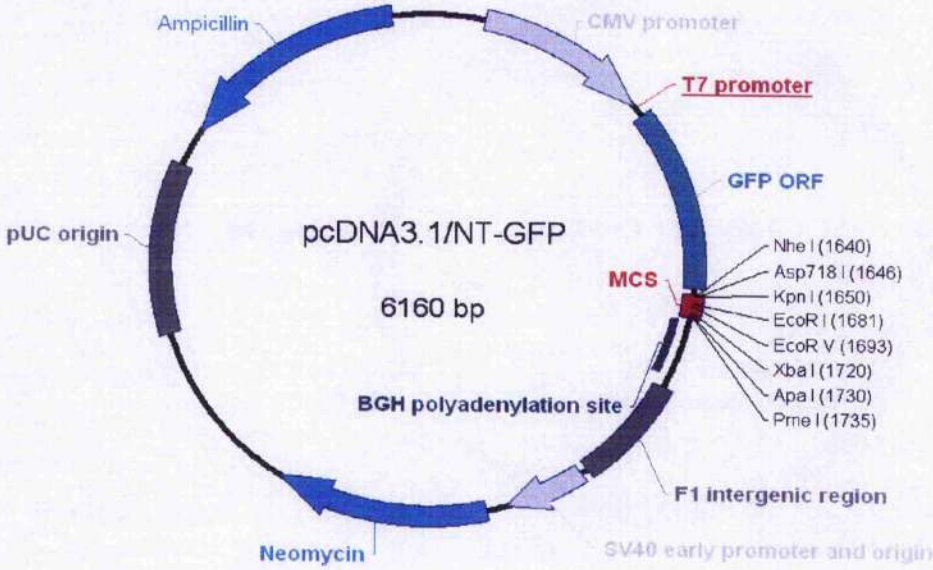


Figure 2.4. *Structure of pcDNA3.1/NT-GFP vector*

2.3- Bioinformatics methods

2.3.1- Resources

NetAffx™ Analysis Center[1]:

This website supported by Affymetrix, integrates array contents and available functional annotations. Its flexible query capabilities allow retrieval of biological information for specific probe sets. The resource provides probe sequence information, gene annotations and gene orthologue and functional information.

NCBI[2]:

The National Center for Biotechnology Information (NCBI) supports and distributes a variety of databases including GenBank, Online Mendelian Inheritance in Man (OMIM), the Molecular Modeling Database (MMDB) of 3D protein structures, the Unique Human Gene Sequence Collection (UniGene), a Gene Map of the Human Genome and the Taxonomy Browser. Its website provides many tools for searching and retrieving information from these databases. Among these tools are Entrez, a retrieval system designed for searching several linked databases, PubMed, a Web search interface that provides access to over 11 million journal citations and sequence analysis tools such as Basic Local Alignment Search Tool (BLAST), Open Reading Frame Finder (ORF Finder) and Electronic PCR. In addition, the NCBI website gives access to genetic and physical maps, among them integrated views of chromosome maps for 17 organisms (MapViewer).

European Bioinformatics Institute[3] (EBI):

The European Bioinformatics Institute (EBI) is a non-profit academic organisation that forms part of the European Molecular Biology Laboratory (EMBL). It manages databases of biological data including nucleic acid, protein sequences and macromolecular structures. Its website provides freely available data together with a large number of bioinformatics tools for similarity and homology searching (BLAST, Fasta), protein functional analysis (InterProScan), sequence (ClustalW) and structural analysis (MSDfold, Dali).

ENSEMBL[4]:

ENSEMBL is a joint project between EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) and the Wellcome Trust Sanger Institute (WTSI) to develop a software system which produces and maintains automatic annotation on selected eukaryotic genomes. Its website presents these analyses in the form of a database that allows different types of queries.

GeneCards®[5]:

GeneCards® is an integrated database of human genes that includes automatically-mined genomic, proteomic and transcriptomic information, as well as orthologies, disease relationships, SNPs, gene expression and gene function information.

Harvester[6]:

The Harvester is an open source software tool created at EMBL-Heidelberg. It caches and cross-links public bioinformatic databases and prediction servers to provide fast access to protein specific bioinformatic information. Implemented databases and servers include: Uniprot/SWISSprot, ENSEMBL, BLAST (NCBI), SOURCE, SMART, STRING, PSORT2, CDART, UniGene and SOSUI.

Different gene prediction programs were used, including Genscan[7], GRAIL[8], HMMGene[9], GeneID[10], and Integrated System for exon finding[11] (Zhang's MZEF + Thanaraj's Splice Proximal Check). In addition two "integrated" programs (GeneComber and NIX) were tried. GeneComber[12] is a graphical interface to the output of several gene prediction algorithms, EUI, GI and EUI-Frame that perform exon and gene structure prediction by taking the intersection of overlapping HMMGene and Genscan predictions. NIX[13] is a tool to view the results of running many DNA analysis programs on a DNA sequence: analysis programs include GRAIL, Fex, Hexon, MZEF, Genemark, Genefinder, FGene, BLAST against many databases, Polyah, RepeatMasker, and tRNAscan.

The ORNL Genome Analysis Pipeline[14] is a pipeline that predicts gene structure from the input sequence and uses this information to then perform motifs and protein predictions.

Uniform Resource Locators (URLs) of all these software or tools can be found in the bibliography.

2.3.2- Homology screening

Comparisons of the rat EST sequences with mouse, rat and human cDNA and genomic DNA databases were performed to identify homologous genes for the ESTs. For each differentially expressed probe set, the exemplar sequence was retrieved using NetAffx™. Exemplar sequences refer to the longest member of an Affymetrix sub-cluster; *i.e.* the longest member of a group of sequences all representing the same transcript; sub-cluster sequences are the building blocks of probe selection.

The principle of the homology screening strategy is that exemplar sequences were individually aligned against different databases. BLAST at ENSEMBL rat database (cDNA and genomic databases) was used to confirm the identity and the chromosomal localisation of the differentially expressed rat genes and the exemplar sequences were then compared to the mouse and human databases (cDNA and genomic) to identify the human and mouse orthologues. Sequences of presumed rat and mouse/human genes were finally aligned at ENSEMBL to check they were genuine orthologues. Appendix A shows screenshots of NetAffx™ and ENSEMBL websites representing each step of this process for a given differentially expressed probe set.

However, because of the nature of the ESTs (which are uncharacterised sequences) and of the lack of rat transcriptome information, the homology screening process often proved to be more complex and involved repeated replicated sequence comparisons, as shown in the flow diagram (figure 2.5).

In most cases, the ESTs remained anonymous rat transcripts, with no rat gene and no mouse/human gene identified by aligning the exemplar sequence against appropriate databases. Identification of the rat gene was sometimes achieved by comparing the rat genomic locus with the rat homologous locus of presumed mouse/human genes and analysing neighbouring known or predicted genes. This process allowed the prediction of novel rat genes or novel exons (*e.g.* novel splice variants).

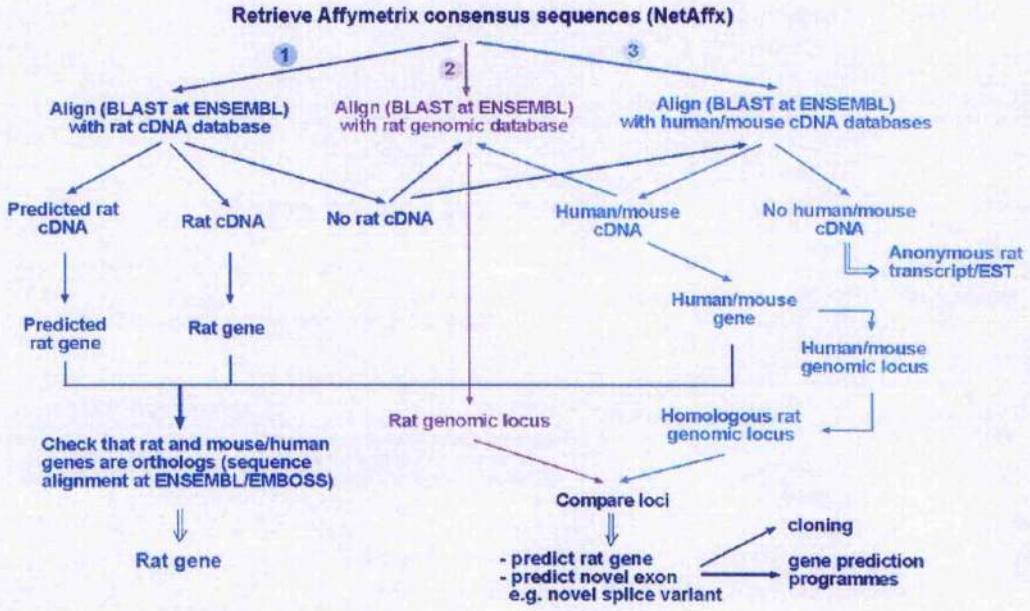


Figure 2.5. *Bioinformatic strategy for homology screening to identify rat genes*

After retrieval of the consensus sequence of each probe set from the Affymetrix website (NetAffx), this sequence was aligned (BLAST at ENSEMBL) first with rat cDNA (1), then with rat genomic (2) and finally with mouse and human cDNA (3) databases, and processed for further analysis in each case.

Although absence of identification of rat cDNA from the rat alignment directly (from 1) prompted rat genomic (2) and mouse/human cDNA (3) alignments, all four alignments were performed for each probe set anyway to check orthology between the genes identified and thereby increase confidence in the rat gene identified by the whole process.

When no rat gene but a rat genomic locus was identified from (2), this locus was compared with that of the rat orthologous gene of potentially identified human and mouse genes (from 3). This information was then used to potentially predict novel rat genes or novel exons of known or predicted rat genes, following what cloning and gene prediction programs were used for further *in vitro* and *in silico* analysis, respectively.

In case not any rat, mouse and human gene was identified after performing all four alignments, the EST represented by the probe set was considered an anonymous rat transcript.

2.3.3- Data mining and selection of candidate genes

GeneCards® and Harvester were used for doing data mining on all differentially expressed ESTs or genes and identify expression patterns, functions and any associations with schizophrenia. Mapping of the genes/ESTs loci with well-

supported regions linked to schizophrenia (reported in many papers, section 1.2.3.2) was also considered.

In addition, the fold changes of the differentially expressed ESTs/genes were investigated and all information was combined and used as criteria for selecting genes that might be worth validating. These candidate genes were then classified into different categories.

2.3.4- Extended bioinformatic analyses on a selected EST

More thorough analyses were performed on a selected EST that had been confirmed as differentially expressed in the rat chronic PCP model.

The facilities BLAST, Entrez, Unigene and dbESTs (NCBI) were used to obtain information about the sequence of this EST and identify other ESTs belonging to the same cluster. ClustalW (EBI) was then used to obtain a multiple alignment of the DNA sequences of these ESTs.

To try to predict a potential novel gene the EST could be part of or identify a novel splice variant of a known gene, integrated programs such as GeneComber and NIX were used as well as several gene prediction programs, including Genscan, GRAIL, HMMGene, GeneID, and Integrated System for exon finding (Zhang's MZEF + Thanaraj's Splice Proximal Check).

2.3.5- Protein prediction software

Position specific iterative BLAST (PSI-Blast) was used for searching protein sequence for similarities with database protein sequences. PSI-BLAST is similar to BLAST except that it uses position-specific scoring matrices derived during the search and may therefore be more sensitive than BLAST (meaning that it might be able to find distantly related sequences that are missed in a BLAST search).

Protein prediction tools were used to extract biologically important information from protein sequence and specifically search for sequence motifs or patterns that might surrender the function of the protein. Many software were used, including ProtFun (Jensen *et al.* 2002; Jensen *et al.* 2003), Blocks (Henikoff and Henikoff 1994), Jpred

(Cuff *et al.* 1998; Cuff and Barton 1999; Cuff and Barton 2000), Pfam (Finn *et al.* 2006), PredictProtein (Rost and Liu 2003), Prosite (Gattiker *et al.* 2002; Sigrist *et al.* 2002), TopPred (von Heijne 1992; Claros and von Heijne 1994).

2.4- Molecular Biology

2.4.1- Preparation and purification of DNA

2.4.1.1-Preparation of plasmid DNA

Preparations of plasmid DNA were performed using Plasmid Mini and Midi kits (Qiagen). DNA was purified from 10ml and 100ml bacterial cultures by alkaline lysis followed by anion-exchange columns, according to the manufacturer's manual.

2.4.1.2- Phenol/chloroform extraction of DNA

Phenol/chloroform extraction was used to further purify DNA (such as plasmid DNA extracted from bacterial cultures).

Briefly, 50% (v/v) phenol 50% (v/v) chloroform was added to the DNA and mixed gently by inversion. The two phases were separated by centrifugation at 12,500 g for 5 minutes and the nucleic acids were precipitated from the upper phase by adding 0.1 volume of 3M sodium acetate and 2 volumes of 100% (v/v) ethanol. After mixing, the tube was incubated on ice for 5 minutes. The nucleic acid pellet were collected by centrifugation at 13,000 g for 15 minutes and washed in 70% (v/v) ethanol. The pellet was air dried and resuspended in TE buffer.

2.4.1.3- Purification of DNA fragments from agarose gels

Gelstar stained-DNA fragments (section 2.4.7) were excised from agarose gels after visualisation with UV light. Extraction of DNA was performed using the QIAquick Gel extraction kit (Qiagen) according to the manufacturer's manual. Briefly, gel

fragments were dissolved in a high-salt buffer, the mixture was applied to a silica-gel membrane column and pure DNA was eluted with low-salt buffer or water.

2.4.1.4- Purification of DNA from PCR reaction mixture

In some cases, the PCR product was directly purified from the PCR reaction mixture. This was only possible when the PCR reaction was determined to have given a single amplicon as checked by loading some of the PCR reaction mixture on an agarose gel and visualising it with UV light. Cleaning up of the PCR mixture was performed using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's manual. As with the QIAquick Gel extraction kit (described previously), this method is based on the adsorbance of nucleic acids to a silica-gel membrane in high-salt conditions and their elution in low-salt conditions.

2.4.1.5- Determination of DNA concentration

DNA concentration was determined by spectrophotometry after appropriate dilution (usually a 1/10 dilution in H_20).

2.4.2- RNA isolation

Qiagen RNeasy Mini kit was used to isolate total RNA from mammalian cell cultures. RNA was isolated by guanidine-isothiocyanate lysis and purified using a silica-gel–membrane according to the manufacturer's manual. Within this protocol, the optional DNase digestion was performed to ensure complete DNA removal (which can be critical in DNA-sensitive applications such as qRT-PCR with a low abundant target).

The quality of the RNA was analysed at the Sir Henry Wellcome Functional Genomics Facility (University of Glasgow) using the Nano LabChip kit on an Agilent 2100 Bioanalyzer.

RNA concentration was determined by spectrophotometry.

2.4.3- cDNA synthesis

First strand cDNA was synthesised from rat brain total RNA using oligo-dT primers. Briefly, 10nmol dNTP and 0.25µg oligo-dT (both from Invitrogen) were added to 1µg total RNA in a RNase-free PCR tube and the volume was adjusted to 12µl with nuclease-free H_2O (Invitrogen). After gentle mixing and a pulse spin the tubes were heated at 65°C for 5 min in a ThermoHybaid PCR thermocycler. 1X buffer, 0.2µmol DTT and 40u RNase out (all from Invitrogen) were then added to each tube and the tubes were heated at 42°C for 2 min. Eventually, 200u Superscript III RT (Invitrogen) was added to each tube and the tubes were heated at 42°C for 50 minutes, followed by 70°C for 15 minutes. First strand cDNA was stored at -20°C.

Human first strand cDNA was synthesised from human brain total RNA following the same protocol as for the rat cDNA except that 6µg random hexamers (Invitrogen) were used for priming the RT reaction and an additional heating step of 10 minutes at 25°C was introduced before the final heatings at 50°C and 70°C. First strand cDNA was stored at -20°C.

2.4.4- PCR

PCR was performed using plasmid DNA to amplify gene fragments for subcloning or cloning. For this purpose, 5' and 3'-specific primers were designed to contain recognition sites for restriction enzymes of the multiple cloning site of the target subcloning or cloning vector so that digestion of the amplified product and of the target vector with the same enzymes followed by ligation will insert the gene of interest in its target vector, in frame if necessary.
PCR was also performed for confirming gene expression in premade cDNA library and for relating an EST sequence to its upstream predicted gene.

In all cases, PCR was performed following a standard protocol using 2-200ng DNA. Reactions were performed in 50µl final volume using 1X Advantage® 2 polymerase, 1X Advantage® 2 PCR buffer, 1X dNTP mix and 0.025nmol of each primer or 1u KOD Hot start polymerase, 1X PCR buffer, 10nmol dNTPs, 50nmol $MgSO_4$ and 0.015nmol of each primer. Annealing and extension temperatures were determined

depending on the melting temperature (Tm) of the primers. 40 cycles were usually performed on a ThermoHybaid PCR thermocycler and a portion of the reaction was loaded on an agarose gel for checking for a single amplicon of the correct molecular weight.

2.4.5- qRT-PCR

Taqman® real-time quantitative PCR (qRT-PCR) was used for validating the differential expression of candidate genes. The first assays were designed manually while the others were purchased as ready-to-use mixes containing specific primers and probe in optimal concentrations (Applied Biosystems). In both cases, the assays were selected as close as possible to the location of Affymetrix probe sets within the ESTs (rat assays) and as close as possible to their human orthologous region (human assays).

For the manually-designed assays, optimal results were obtained through a careful experimental design, including selecting the appropriate enzymes, designing optimal primers, using different buffers and additives, establishing cycling parameters and preparing high quality templates.

Primer Express™ software package (Applied Biosystems) was used for designing specific PCR primers and fluorescently labelled probe).

Primers specificity was checked by performing a RT-PCR using qRT-PCR primers and rat/human brain first strand cDNA. Single amplicons of the right molecular weight were then sequenced to confirm gene identity using the forward and reverse qRT-PCR primers as sequencing primers.

Primers and probe concentrations were optimised by trying different dilutions of each component and the conditions giving the most efficient reaction were chosen.

The optimised qRT-PCR assay was then performed using dilutions of rat/human brain first strand cDNA to check for efficient amplification with different amounts of first strand cDNA.

Real-time PCR was eventually performed on target gene and endogenous control gene with rat or human brain samples.

For both types of assays (manually-designed or Assays-on-Demand®), qRT-PCR was performed using an ABIPrism 7000 (Applied Biosystems) in the Absolute quantitation mode following the manufacturer's protocol and using the recommended PCR programme consisting of 10 min initial denaturation at 95°C followed by 40 cycles for 15 sec at 95°C and 1 min at 60°C.

Beta-2-microglobulin (*B2m*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), which had previously been determined as the most appropriate housekeeping genes to use in these conditions (they were shown to exhibit reliably unchanged expression in the chronic PCP model of schizophrenia and in post-mortem prefrontal cortex from schizophrenic patients, Hiromitsu Ozeki) were used for the rat and human qRT-PCR assays respectively. A standard curve consisting of accurate first strand cDNA dilutions was performed in some cases. H_2O negative controls were added to each reaction plate to ensure the absence of contamination.

Analysis of covariance (ANCOVA) was used to determine the effect of chronic PCP treatment on the expression level of candidate genes. This approach allows partialling out any effects of PCP treatment on the expression levels of housekeeping genes from those specific to the candidate gene of interest and harbours the potential to increase the statistical power of qRT-PCR analyses (Bond *et al.* 2002). For the analysis, four cycles were chosen for each gene from the linear phase of amplification and the model incorporated 'treatment group' (PCP or vehicle), 'cycle number' (n=4), and 'subject' (n=5 or 6 samples) as the independent variables with 'subject' included as a random factor nested within the treatment group variable. Expression levels of the housekeeping gene were incorporated as the covariate. As the ANCOVA analysis relies on the assumption that regression slopes are homogeneous, interaction between the dependent variable and the covariate was investigated and proper ANCOVA analysis of the data was only performed when having established that the regression slopes assumption had not been violated.

2.4.6- RACE PCR

Rapid Amplification of cDNA Ends (RACE) PCR was used for extending an EST sequence in the 5' direction. Rat brain Marathon-Ready cDNA (Clontech) was used as a template for cDNA amplification. It consists of a premade "library" of adaptor-

ligated double-stranded cDNA ready for use as template in both 5'- and 3'-RACE PCRs.

RACE PCR was performed as recommended by Clontech. An EST-specific reverse primer was designed to be the starting point for DNA elongation in the 5' direction. This primer was similar to the reverse primer used for qRT-PCR but it was redesigned to comply with the criteria for RACE PCR and for use with Clontech adapter specific Primer. It was used together with an Adaptor Specific primer (provided by Clontech).

As recommended by the manufacturer, several controls were run alongside the experimental sample: two negative controls with only one primer each (either the Adaptor-specific primer or the EST-specific primer), one positive control for amplifying *GAPDH* using the Adaptor-specific primer and a control *GAPDH* 5'RACE primer (Clontech) and one positive control for amplifying the EST using the EST-specific primer and the forward primer used in qRT-PCR. Negative controls without any template were also run to check there was no contamination.

Reactions were performed in 50µl total volume using 0.5ng Marathon-Ready cDNA, 0.2µM of each primer, 1X cDNA PCR reaction buffer, 0.2mM dNTP mix and 1X Advantage 2 Polymerase Mix (in water).

Thermal cycling was performed using the following programme (optimised for the EST-specific primer): 1 min initial denaturation at 94°C followed by 30 cycles for 30 sec at 94°C and 4 min at 68°C.

5µl (out of 50µl PCR reaction) of each reaction were analysed in a 1.2% (w/v) agarose gel stained with Gelstar nucleic acid along with 0.1µg 1Kb+ DNA ladder.

2.4.7- Agarose gel electrophoresis

DNA molecules were separated according to size by agarose gel electrophoresis. Solutions of 1-2.5% (w/v) agarose in 1X TBE were prepared in a microwave. When the solution had cooled to approximately 50°C, 1/10,000 dilution Gelstar nucleic acid stain was added and the gel cast in a horizontal tray with a 1.5mm comb. DNA samples were mixed with 1X BlueJuice gel loading buffer and subjected to electrophoresis in 1X TBE for 2 to 4 hours at 80-100 volts/cm. 0.1µg 1Kb Plus DNA ladder (Invitrogen) was also run as a DNA size marker.

Separated DNA samples were visualised using a UV transilluminator (wavelength 254nm) and photographed.

2.4.8- SNP genotyping

For SNP genotyping, 2ng genomic DNA were used per allelic discrimination assay in a total volume of 10µl which had been determined to give as accurate results as the 25µl final volume recommended by Applied Biosystems.

1µl DNA (at 2ng/µl concentration) was delivered per reaction to the bottom surface of an optical reaction plate and 9µl SNP reaction mix (1.25µl SNP genotyping assay mix, 5µl 2X TaqMan Universal PCR Master Mix, and 2.75µl DNase-free water per reaction) were added to each well. Plates were covered and sealed with an optical adhesive cover and centrifuged briefly to collect all reagents at the bottom of the wells and eliminate all eventual air bubbles.

SNPs genotyping was performed on an ABIPrism 7000 in real-time (absolute quantitation) and end-point reading (allelic discrimination) modes following the manufacturer's protocol and using the recommended PCR programme. Real-time mode was run first to allow troubleshooting if needed and end-point reading mode allowed calling of the alleles for genotyping. Every single sample's genotype was checked manually to ensure accurate results.

2.4.9- DNA cloning technique

2.4.9.1- Restriction endonuclease digestion of PCR-amplified DNA fragments

Restriction digests were carried out to generate gene fragments for subcloning and for checking the inserts of recombinant plasmids containing foreign DNA.

250ng-1µg of Qiagen-prepared plasmid DNA or PCR-amplified DNA, 1-2 units of restriction endonuclease, 1X recommended restriction endonuclease buffer in a total volume of 20 or 50µl with H_20 were incubated at 37°C overnight.

2.4.9.2- DNA ligation

Bacteriophage T4 DNA ligase was used to catalyse the formation of phosphodiester bonds between the 3' hydroxyl groups and the 5' phosphate groups of the restriction-digested DNA inserts and vectors. Ligation of vector and insert DNA were incubated at 4°C overnight with 1 unit of bacteriophage T4 DNA ligase and 1X T4 DNA ligase buffer in a total volume of 10µl. DNA molecules were ligated at a vector:insert molar ratio of 1:3. Control ligations, with one component missing, were also incubated at 4°C overnight.

2.5- Cellular biology

2.5.1- Transformation of competent bacterial cells

200µl of DH5α or TOP10 competent cells were thawed on ice for 10 minutes. 1µl of the ligation mixture (section 2.4.9.2) or 10ng of control plasmid DNA was added to the competent cells and the tube was gently mixed. After 10 minutes incubation on ice, the cells were heat-shocked at 42°C for 1 minute and immediately placed on ice for 2 minutes.

800µl of chilled SOC medium (a specialised microbiological medium which is a derivative of SOB –Super Optimal Broth- medium differing in that it contains glucose therefore gives rise to Catabolite repression) was added to the cells and incubated at 37°C for 1 hour.

Cells were centrifuged for 3 minutes at 10,000g, resuspended in 200µl LB and spread on an LB agar plate containing 100g/ml ampicillin (to positively select transformed bacteria). The plates were incubated at 37°C overnight.

2.5.2- Generation of plasmid stocks

Stocks of bacterial host cells and bacteria transformed with recombinant plasmid were prepared for long term storage.

A colony from an agar plate was used to inoculate 10ml (Mini Prep) or 100 ml (Midi Prep) of LB containing 100µg/ml ampicillin (for transformed bacteria) and shaken at 37°C overnight. 800µl of the overnight culture were mixed with 200µl of 50% (v/v) glycerol 50% (v/v) LB, mixed and stored at -70°C.

2.5.3- Mammalian cell culture

Human SK-N-SH cells were cultured in D-MEM with L-glutamine, 4500mg/L D-glucose and 110mg/L sodium pyruvate supplemented with 10% (v/v) FBS.
Rat PC12 cells were cultured in D-MEM with GlutaMAX™, 4500mg/L D-glucose and without sodium pyruvate supplemented with 10% (v/v) FBS. Differentiation was initiated by the addition of 50ng/ml nerve growth factor (murine NGF 2.5S, Invitrogen).
Rat C6 glioma cells were cultured in Ham F12 medium supplemented with 10% (v/v) FBS and 1mM glutamine.
All cells were maintained at 37°C with 5% CO_2.

In all cases, confluent cells were washed with PBS at room temperature and detached with 0.05% (w/v) trypsin-EDTA (2mls for 75cm^2 flasks and 400µl for 25cm^2 flasks) at 37°C in the incubator.
The reaction was stopped by the addition of an excess of medium with FBS and cells were centrifuged for 5min at 1000g. Pelleted cells were resuspended in their appropriate medium and split into sterile flasks using a ratio to total volume of cell suspension of 1:6 for SK-N-SH and PC12 cells and 1:5 for C6 cells. Volume was adjusted to 10ml for 25cm^2 flasks and 25ml for 75cm^2 flasks.
Trypan blue was used for counting cells when they had to be seeded at a certain density (before transfection for example). Haemocytometer chambers were filled with the cell sample diluted in trypan blue exclusion solution. Live cells present in each of 10 squares (1mm^2, delimited by double lines, 9 small squares) were counted after a few minutes (to allow their deposition on the counting plane) using a microscope with 10X objective. The number of cells per ml was calculated by multiplying the average count per square by 10^4 and by the dilution factor used for diluting cells in trypan blue.

For making stocks of early passage cells, pelleted cells obtained after trypsinisation and centrifugation were resuspended in FBS with 1% (v/v) DMSO and frozen at -80°C as gently as possible (cryotubes were wrapped with cotton and placed inside polystyrene boxes to slow down the freezing process).

2.5.4- Transfection of cells

2.5.4.1- Transfection of SK-N-SH and C6 cells

TransIT®-LT1 transfection reagent (Mirrus Bio Reagents) was used for transfecting SK-N-SH and C6 cells with plasmid DNA.

Approximately 24h prior to transfection, the cells were plated at a cell density of 1-2 x 10^5 cells in complete growth medium per well of a 6-well plate, to obtain 50-70% confluence the following day, and were incubated overnight at 37°C in 5% CO_2. 24h later, 6µl TransIT®-LT1 transfection reagent were added into 250µl of serum-free medium in a sterile plastic tube. After 20 minutes incubation at room temperature, 2µg plasmid DNA were added to the diluted transfection reagent and incubated for 20 minutes at room temperature. In the meantime, medium was removed from the cells and replaced with fresh complete growth medium. The transfection reagent/plasmid DNA complex was then added dropwise to the cells in complete growth medium.

The plate was rocked gently back and forth and from side to side to distribute the complexes evenly. Cells were incubated for 24-48h depending on the application.

2.5.4.2- Transfection of PC12 cells

Lipofectamine™ 2000 reagent (Invitrogen) was used for transfecting PC12 cells.

Approximately 24h prior to transfection, PC12 cells were plated at a cell density of 4*10^5 cells in complete growth medium per well of a 12-well plate so that they are 90% confluent on the day of transfection.

For each well of cells to be transfected, 1.5µg DNA was diluted in 100µl of Opti-MEM® medium. 4µl of Lipofectamine® 2000 reagent was diluted in another 100µl of Opti-MEM® and incubated for 5 minutes at room temperature. The diluted reagent was then combined with the diluted DNA and incubated for 20 minutes at room

temperature. DNA-Lipofectamine® 2000 complexes (200µl per well) were added directly to each well and the plate was rocked gently back and forth and from side to side to distribute the complexes evenly. After 24h incubation at 37°C, normal proliferation medium was replaced by differentiation medium (D-MEM supplemented with 50-100nm/ml NGF) and the cells were cultured for 1 to 6 days.

2.5.5- LPA treatment

For assessing the effects of LPA treatment on biochemical pathways, cells were treated with LPA prior to total protein extraction. Briefly, cells were washed with PBS and cultured for 30 minutes in serum-free medium containing LPA (at the desired final concentration, usually 1µM) or vehicle (PBS with 0.1% (w/v) BSA). Proteins were then extracted after washing with PBS (section 2.5.6).

2.5.6- Cell lysis

Modified RIPA buffer was used to extract proteins from adherent cells.
Briefly, fresh ice-cold RIPA buffer containing protease inhibitors was added to cells washed with PBS and plates were left on ice for 15 minutes. After this incubation, cells were scraped and cell suspension transferred into an ice-cold eppendorf was centrifuged at 13,000g for 15 minutes at 4°C. Resulting supernatant was transferred into another clean ice-cold eppendorf and proteins were aliquoted and stored at -20°C.

2.5.7- Membrane preparation

Membrane proteins were extracted from cells for performing the GTPγS-binding assay.
After overnight starvation in serum-free medium, cells were washed with PBS, scraped in 20mM Hepes, 1mM EDTA, 50mM NaCl (pH 7.5) and homogenised with a pastette.
A first centrifugation at 1,000g for 5 min was performed to remove cell debris and the supernatant was further centrifuged at 40,000g for 1 hour at 4°C. Pellet was

resuspended in ice-cold 50mM Hepes, 2mM EDTA, 100mM NaCl, 1mM $MgCl_2$ and aliquots were stored at -80°C.

2.5.8- Protein assay quantification

Protein concentration was measured using the dye-binding assay of Bradford.
Bradford reagent (Biorad) was diluted five fold in H_2O (1 part Bradford reagent:4 parts H_2O) and diluted reagent was filtered through a 0.2µM filtration unit.
A standard curve was prepared using a serial dilution series (31.25ng/µl to 250ng/µl) of a bovine serum albumine sample.
In a 96-well plate, 200µl of diluted reagent was added in duplicates to each protein standard and each protein sample (usually diluted 1/10 in H_2O). Absorption was measured after 10 minutes at a wavelength of 595nm using a spectrophotometer.

2.5.9- Western Blot

2.5.9.1- Polyacrylamide gel electrophoresis

Proteins were analysed using dissociating, discontinuous SDS-PAGE. Electrophoresis was carried out in mini separating precast 10% (w/v) Tris-HCl gels containing a 10% (w/v) resolving gel and a 4% (w/v) stacking gel in BIO-RAD Mini Protean III gel apparatus. 0.75mm thick mini gels with 10 and 15 wells were used.
Protein samples were mixed with 3X SDS-PAGE loading buffer or 5X SDS-PAGE loading buffer, boiled for 5 minutes at 100°C and subjected to electrophoresis in 1X SDS-PAGE running buffer at 70mA with maximum voltage for 1 hours to 3 hours, depending on the protein samples to be separated. 6µg of kaleidoscope prestained protein standards (BIO-RAD) were subjected to electrophoresis as protein size markers.

2.5.9.2- Western blotting

Proteins resolved by SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membrane for western analysis.

Polyacrylamide gels and filter paper were equilibrated in western blot transfer buffer (20mM Tris, 150mM glycine, 20% (v/v) methanol) for 15 minutes. PVDF membrane was wetted in methanol and also equilibrated in transfer buffer for 15min. The blotting apparatus was then sandwiched together and placed in a tank of cold transfer buffer with a block of frozen transfer buffer to facilitate keeping the unit cool. 100v were passed through for 1 hour 30 minutes and membranes were used immediately for western analysis

2.5.9.3- Immunodetection

Proteins resolved by SDS-PAGE and transferred onto PVDF membranes were analysed by immunodetection. PVDF membranes were rinsed in 1X TBS then blocked in blocking buffer (1X TBS/0.1% (v/v) tween 20/5% (w/v) skimmed milk powder) at room temperature for 1 hour. The blocking solution was discarded and the membranes were washed 5 times for 5 minutes in 1X TBS/0.1% (v/v) tween 20 at room temperature. The primary antibody was diluted to the optimum concentration (either recommended by manufacturer or previously determined experimentally, see Table 2.7) in 5 or 10ml of 1X TBS/0.1% (v/v) tween 20/1% (w/v) skimmed milk powder per 8.6x6.8cm² membrane. Diluted antibody was added to the membrane and incubated at room temperature for 1 hour or overnight at 4°C. The membranes were washed again in 1X TBS/0.1 (v/v) tween 20. The secondary HRP-conjugated antibody was optimally diluted (Table 2.6) in 10ml of 1X TBS/0.1% (v/v) tween 20/1% (w/v) skimmed milk powder per 8.6x6.8cm² membrane. The diluted secondary antibody was added and incubated at room temperature for 1 hour. The 1X TBS/0.1 (v/v) tween 20 washes at room temperature were repeated as before. Detection reagents from the ECL™ Plus western analysis system (GE Healthcare, formerly Amersham Biosciences) were used according to the manufacturer's protocol to detect the signal produced by the antigen-antibody-antibody interactions. Detection reagents A and B were equilibrated at room temperature and mixed (50µl of reagent B with 2ml of reagent A) so that the final volume was 0.035ml/cm² of membrane. The membranes were covered with the detection solution for 5 minutes at room temperature and after removal of excess solution they were sealed in cling film wrap and exposed to autoradiographic film for 10 seconds to 1 hour.

Supersignal west femto maximum sensitivity substrate (Pierce Biotechnology) was tried as an alternative to the ECL™ Plus western analysis system. Working solution (0.1ml per cm^2 of membrane) of substrate for detecting HRP on immunoblots was prepared by mixing equal parts of the stable peroxide solution and the luminol/enhancer solution. Membranes were covered with the detection solution for 5 minutes at room temperature, drained, sealed in cling film wrap and exposed to autoradiographic film for the appropriate time.

Antibody	Dilution	Incubation
Rabbit anti-actin	1:1000	Overnight, 4°C
Mouse phospho-SAPK/JNK	1:2000	Overnight, 4°C
Actin HRP-linked	1:1000	1 hour, room temperature
Anti-FLAG HRP-linked	1:5000	1 hour, room temperature
Anti-mouse HRP-linked	1:1000	1 hour, room temperature
Anti-rabbit HRP-linked	1:2000	1 hour, room temperature

Table 2.7. *Dilutions and conditions of use of antibodies for western blottings*

2.5.9.4- Autoradiography

Membranes from western analyses were sealed in cling film wrap and exposed to X-ray film in cassettes containing intensifying screens. PVDF membranes were incubated from 10 seconds to 30 minutes at room temperature. The autoradiographic film was developed using an automatic developer (Hyperprocessor, Amersham Life science).

2.5.10- Enzyme-linked immunosorbent assay (ELISA)

A Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA Kit (Cell Signalling Technologies) was used for measuring JNK activation in protein samples.

In this assay, microwells have been coated with a SAPK/JNK Mouse antibody. After incubation with cell lysates (extracted with the cell lysis buffer provided with the kit), the coated antibody captures both non phospho- and phospho-SAPK/JNK proteins. Following extensive washing, a phospho-SAPK/JNK antibody is added to detect the captured phospho-SAPK/JNK protein. HRP-linked anti-rabbit antibody is then used to recognise the bound detection antibody and an HRP substrate,

tetramethylbenzidine (TMB), is added to develop colour. The magnitude of optical density for this developed colour is proportional to the quantity of phospho-SAPK/JNK protein.

For this assay, 20µg protein was used in duplicate and the first incubation of the sample in coated microwells was performed overnight at 4°C. The assay was performed according to the manufacturer's protocol and consists of a series of incubations and extensive washings with the appropriate provided buffers. The optical density of the developed colour was measured at 450nm (using a spectrophotometer) within 5 minutes after the addition of STOP solution.

2.5.11- Immunoprecipitation

Immunoprecipitation was used for isolating proteins that complex with KIAA1189 and therefore potentially interact with it. The N-terminal FLAG tag cloned in fusion with KIAA1189 allowed affinity purification and immunoprecipitation of the complex using a highly specific antibody.

FLAG M2 immunoprecipitation was performed using a dedicated kit (Sigma-Aldrich) following the manufacturer's protocol. Briefly, FLAG-KIAA1189-overexpressing cells were rinsed with PBS and lysed. After centrifugation, the supernatant consisting of proteins was transferred to a chilled tube and stored at -80°C if not used straight away.

Anti-FLAG M2 agarose affinity gel (40µl per reaction) was thoroughly resuspended and the resin was washed twice with 0.5ml wash buffer. In order to remove any traces of unbound ANTI-FLAG antibody from the resin suspension, the resin was washed once with 0.5 ml elution buffer before to be washed again three times with 0.5ml wash buffer.

Cell lysate was added to the resin and final volume was brought up to 1ml with lysis buffer. For the positive control, 200ng of a FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP) control protein (provided) was added to the resin; for the negative control 1ml of lysis buffer with no protein was added to the washed resin. Samples were agitated using a circular rotor overnight at 4°C.

The resin was briefly centrifuged and then washed three times with 0.5ml of wash buffer carefully removing all the supernatant each time.

Elution was performed under native conditions by a competition with a 3X FLAG peptide (provided). 100µl of elution buffer containing 150ng/µl 3X FLAG peptide were added to the resin in each test tube. Samples and controls were incubated with gentle shaking for 30 minutes at 4°C. The resin was then centrifuged and supernatants containing protein complexes were transferred to fresh test tubes.
Subsequent detection of proteins was performed by loading samples on an SDS-PAGE gel and immunoblotting with specific antibodies (anti-FLAG M2 antibody for the positive control).

2.5.12- Immunofluorescence

Indirect immunofluorescence was used to investigate endogenous or exogenous protein expression in different cell types.
Prior to the experiment, 13mm diameter coverslips were placed in 12- or 6-well plates and coated with poly-D-lysine by adding diluted poly-D-lysine for 10 min, washing with water, then air drying. Cells were grown on these coverslips as usual.
On the day of the experiment, cells were brought to room temperature, washed with PBS and fixed with 4% (w/v) paraformaldehyde for 10 minutes at room temperature. After three 5 minutes washes with PBS, cells were permeabilised for 5 minutes with 0.5% (v/v) Triton X-100 in PBS and washed again with PBS.
Non-specific adsorption of the antibodies to the coverslip was prevented by first covering the coverslips with blocking buffer (0.1% (v/v) Triton X-100 in PBS with 1% (w/v) BSA) for 1 hour at room temperature.
After three 5 minutes washes with PBS, 10-50µl of primary antibody (diluted in blocking buffer) was applied on each coverslip and incubated for 1 hour at room temperature or overnight at 4°C. For double immunofluorescence procedures, cells were incubated simultaneously with two separate primary antibodies (in this case, the antibodies must be monospecific and raised in different species). The concentration of antibody to be used depends on the affinity of the antibody and the abundance of the antigen. Concentrations and incubation conditions of all the antibodies used are listed in table 2.8.
Coverslips were washed three times 5 minutes with PBS before being incubated with secondary antibodies conjugated to a fluorochrome, depending on the donor species of the primary antibody. Cross-adsorbed and affinity-purified secondary antibodies

were used to minimise background, cross reactivity (in double immunofluorescence protocols) and non-specific reactivity from the secondary antibodies. Again, secondary antibodies were diluted in blocking buffer to the appropriate concentration and coverslips were incubated with diluted antibodies for 1 hour at room temperature.

In some cases, coverslips (washed three times 5 minutes with PBS) were incubated with 166nM Alexa Fluor® 488 phalloidin (in PBS) for 20 minutes at room temperature to label F-actin.

Finally, 4',6-diamidino-2-phenylindole (DAPI) was used for staining cell nuclei: after further washings, coverslips were covered with a 300nM DAPI solution and incubated for 5 minutes at room temperature.

Coverslips were washed extensively with PBS, rinsed once with water and each coverslip was inverted onto a slide containing 10µl of mounting media (Vectashield, Vector Labs).

The edges of each coverslip were sealed with regular transparent nail polish and allowed to dry for 5 minutes to provide semi-permanent preparations.

Observation was done using a Nikon microscope equipped with filters for DAPI and for emission at 488 and 596nm.

Antibody	Dilution	Incubation
Mouse monoclonal anti-FLAG M2	1:2000	1 hour, room temperature
Rabbit polyclonal anti-(pan)-actin	Add 200µl 50/50 Gly/H2O MilliQ and use 1:200	1 hour, room temperature
Rabbit polyclonal anti-(pan)-cadherin	2µg/ml	1 hour, room temperature
Rabbit polyclonal to MAP2	1:1000	Overnight, 4°C
Rabbit polyclonal to GAP43	1:300	Overnight, 4°C
Polyclonal anti-rabbit IgGs conjugated with Alexa Fluor® 488	1:200	1 hour, room temperature
Polyclonal anti-mouse IgGs conjugated with Alexa Fluor® 596	1:400	1 hour, room temperature

Table 2.8. *Dilutions and conditions of usage of antibodies for immunofluorescence*

2.5.13- Neurite outgrowth assay

A potential role of KIAA1189 in neurite extension was investigated using PC12 cells overexpressing KIAA1189 and undergoing NGF-induced differentiation.

Prior to the experiment, 13mm diameter coverslips were placed in 12-well plates and coated with collagen (rat tail collagen I, Upstate) by covering the surface of the coverslips with 10μg/cm^2 of collagen I diluted in PBS and incubating for 30 minutes at room temperature.

PC12 cells were grown on these coverslips as usual and co-transfected with either the FLAG-KIAA1189 construct or the empty pcDNA3.1-FLAG expression vector together with GFP (pcDNA3.1/NT-GFP, Invitrogen) using Lipofectamine® 2000 reagent (section 2.5.4.2). The molar ratio of FLAG-KIAA1189 or FLAG alone to GFP construct was 4:1. One day after transfection, the medium was replaced for cell differentiation medium. Six days after differentiation, cells were fixed with 4% (w/v) paraformaldehyde and permeabilised with 0.5% triton X-100, followed by nuclear counterstaining with DAPI (section 2.5.12). GFP epifluorescence was used as a marker for cell morphology. Cell images were captured using a confocal microscope.

Because manual examination of cell morphology is quite a subjective method for measuring neurite outgrowth, a neurite outgrowth quantification assay kit (Chemicon) was used for semi-quantitatively evaluating neurite extension in differentiated KIAA1189-overexpressing PC12 cells in comparison with differentiated PC12 cells transfected with the empty pcDNA3.1-FLAG vector.

Prior to initiation of neurite outgrowth assay, membranes on underside of chambers were coated with collagen I by adding each insert into a well of a 24-well plate containing 500μl of 10μg/ml collagen I and incubating for 2 hours at 37°C. In the meantime, PC12 cells were removed from culture flasks using trypsin, a cell viability assay was performed using trypan blue and the cells were resuspended in non-differentiating medium at 0.5x10^5 cells/ml. Subsequently, each membrane was removed from collagen I coating solution, placed into a well of a 24-well plate containing 500μl non-differentiation medium and a cell suspension volume of 100μl was added on top of membrane (upper chamber).

One day after plating, PC12 cells were transfected with either the FLAG-KIAA1189 construct or the empty pcDNA3.1-FLAG expression vector using Lipofectamine®

2000 reagent (reaction detailed in chapter 2.5.4.2 was scaled down to the surface of the membrane).

One day after transfection, differentiation was initiated by adding 100ng/µl NGF to the medium and neurites were allowed to extend to the lower chamber for 1 to 7 days at 37°C.

2.5.14- GTPγS binding

The GTPγS binding protocol detailed below was adapted from (Im *et al.* 2000) and required optimisation to obtain reproducible results.

25µg of membranes from native SK-N-SH cells or *EDG2* DNA stably transfected SK-N-SH cells were incubated in 1.0ml of GTP-binding buffer (50mM Hepes, 100mM NaCl, 10mM $MgCl_2$, pH 7.5) containing 25µg saponin, 10µM GDP, 0.1nM [γ-^{35}S]GTP (specific activity>1000Ci/mmol) and 0.1nm-10µM LPA for 30 minutes at 30°C.

Bound GTPγS was separated from unbound using a cell harvester (Brandel Research and Development Laboratories) and GF/B filters were washed three times with 20mM Tris, 120mM NaCl, 25mM $MgCl_2$. Filters were immersed in 5ml scintillation solution (Ecoscint A, National Diagnostics) and radioactivity was counted by liquid scintillation spectrometry.

For data analysis, background filter counts, *i.e.* residual radioactivity bound to the filter in the absence of membrane, were subtracted from each sample count and the data were normalised to the maximal binding displayed by SK-N-SH cells when stimulated with the highest dose of LPA.

Dose-response curves were plotted and analysed by non-linear regression analysis with a sigmoidal dose-response curve fitting (GraphPad Prism).

Percentage of increase in binding above basal was calculated to give a measure of efficacy (E_{max}).

2.6- *In situ* hybridisation

Radioactive semi-quantitative *in situ* hybridisation was used to assess differential expression of candidate genes in brain sections from PCP-treated rats compared to vehicle-treated controls.

2.6.1- Probe design

45-mer oligonucleotide probes of unique sequence were designed and aligned against the whole genome to check gene specificity. HPLC-pure oligonucleotides were synthesised by Sigma-Genosys.
The probe sequences are listed in table 2.9.

Probe	Sequence
EST – 2as	GACAAGGAGGTTCTTTATTTAACAGCAGGTAGGTAGGTTGATAAT
Edg2 – 3as	GACACAAGCCACACCCTGCCTTTCCTCCTCCCCAGAATGCCACTC
Q9ULN1 – 2as	TCAGTTGTCTTCCTGGTTTGACTGCTAGGCTGTTGGCCATTCTCA

Table 2.9. *Sequences of probes used for in situ hybridisation*

2.6.2- Oligonucleotide labelling

The probes were 3'end-labelled with α-[^{35}S]dATP (specific activity 1250Ci (46.2TBq)/mmol)) using Terminal Transferase enzyme (Roche). In a sterile eppendorf, the following solutions were mixed together: 2µl DEPC water, 0.9X TdT tailing buffer (Boehringer Ingelheim), 0.6µl CoC12, 0.6pmol oligonucleotide, 2.5µl [α-[^{35}S]dATP] and 2µl TdT enzyme. The reaction mixture was incubated in a water bath for 1 hour at 37°C. The reaction was inhibited by the addition of 60µl DEPC-treated water. Labelled oligonucleotides were purified by using QIAquick™ Nucleotide Removal kit (Qiagen) according to the manufacturer's protocol. 2µl of final eluate containing the labelled probe was analysed by liquid scintillation. Probes with an activity of between 100,000 and 300,000 DPM/µl were considered to be successfully labelled. Subsequently 20mM DTT was added to the probe, which was then stored at -20°C until required.
Oligonucleotides that were successfully labelled were hybridised against test sections as outlined in section 2.6.4 to check for specificity and determine the level of

background binding of the probe. This also enabled us to evaluate the time needed to get a measurable signal, strong enough to quantify gene expression and to compare PCP-treated rats and controls.

2.6.3- Slide preparation and fixation (Sandie MacGregor)

Collection and preparation of coronal sections was performed by Sandie MacGregor. Briefly, 10µm coronal sections were cut on a cryostat at –20°C (Leica) and collected onto poly-L-lysine-coated slides. Sections were obtained from the following bregma levels according to Paxinos (Paxinos 1998). 3.20mm (medial frontal cortex); 1.60mm (nucleus accumbens / striatum); -1.40mm (anterior thalamus); -2.30mm (midline thalamus); -4.80mm (hippocampus). Sections were air dried at room temperature, and once dry, they were fixed in freshly prepared, ice-cold, 4% (w/v) paraformadehyde in phosphate-buffered saline (PBS) for 5 min. After rinsing in PBS for 5min, the sections were dehydrated by consecutive 5 min immersions in 70 % (v/v), 95% (v/v) and 100% ethanol.

For investigating region-specific gene expression changes between PCP-treated rats and controls, one set of sections representing the five layers of the PFC and corresponding to 16 PCP-treated animals and 16 control animals was used per gene.

2.6.4- Hybridisation and controls

In situ hybridisation was performed according to the methodology of Wisden and Morris (2002). Briefly, sections were removed from the fridge and allowed to air dry for about 30 minutes. Hybridisation mixture was prepared for experimental sections by adding 4µl labelled probe and 16µl 1M DTT to 200µl hybridisation buffer (1x SSC, 25ml deionised formamide, 1.25mmol sodium phosphate, 50µmol sodium pyrophosphate, 5mg polyadenylic acid, 5g dextran sulphate) per experimental slide. For control sections, a 4 fold excess of cold oligonucleotide (*i.e.* 16µl unlabelled probe) was added to the hybridisation mixture. Slides were placed in petri dishes with tissue soaked in 4X SSC to prevent the sections drying out, and 200µl of well-mixed hybridisation mixture was applied to each slide. The slides were then covered with parafilm for allowing spreading the mixture so that it covers all the sections and

potential air bubbles were eliminated by gently pressing parafilm. Petri dishes were sealed with parafilm and placed in the incubator at 42°C overnight.

2.6.5- Washings

Parafilm coverslips were removed in 1x SSC at room temperature and slides were transferred into 1x SSC prewarmed at 60°C and incubated with gentle agitation for about 30 minutes. Sections were then transferred through a series of washes, 1X SSC, 0.1X SSC, 70% ethanol and 95% ethanol at room temperature for approximately 20 seconds each, agitating slightly. Sections were allowed to dry for about 30 minutes and slides were placed in X-ray cassettes and exposed to Biomax™ MR film (Kodak) for between 1-6 weeks.

2.6.6- Film development and image analysis

Films were developed after an appropriate time using an automatic developer (Hyperprocessor, Amersham Life science).The films were analysed using computer-based densitometry (MCID 5). Brain regions identified were anatomically defined as outlined in a rat brain atlas (Praxinos and Watson, 1998). Bilateral relative optical density measurements were taken in discrete brain sections from duplicate sections from each animal (n=16 per treatment group). The results were analysed using individual t-tests for each brain region to assess the differential expression of the genes after PCP treatment. Statistical significance was defined as $p < 0.05$.

CHAPTER 3: IDENTIFICATION, SELECTION AND VALIDATION OF DIFFERENTIALLY EXPRESSED GENES

3.1- Introduction

A global transcriptome screen to identify differentially expressed novel psychosis-related genes was performed in our laboratory utilising a phencyclidine (PCP) model of schizophrenia and rat oligonucleotide GeneChips® (rat genome U34 set) from Affymetrix (data provided by Catherine Winchester). In summary, rats (n=6 per group) were treated chronically with PCP or vehicle according to the regime described in Cochran *et al.* (2003). The PFC was dissected and total RNA extracted using the RNeasy Miniprep Kit (Qiagen). Biotin-fluorescein labelled cDNA was synthesised according to the Affymetrix protocol and hybridised to RG-U34 A, B and C GeneChips®. Pre-processing (background correction, normalisation, combination of probe set data into a single expression measurement and log2 transformation) of the GeneChip® data was performed using RMA (Robust Multi-Array Average Expression Measure, Bioconductor) and Significance Analysis of Microarrays (SAM) was used to perform a high-level statistical analysis of the normalised GeneChip® data to identify differentially expressed probe sets. After stringent normalisation and rigorous statistical analyses, 118 differentially expressed probe sets were identified from the RGU34A GeneChip® data and 209 differentially expressed probe sets were identified from the RGU34B and C GeneChip® data. The microarray study allowed genes and also expressed sequence tags (ESTs) that have the potential to represent novel and uncharacterised genes to be screened.

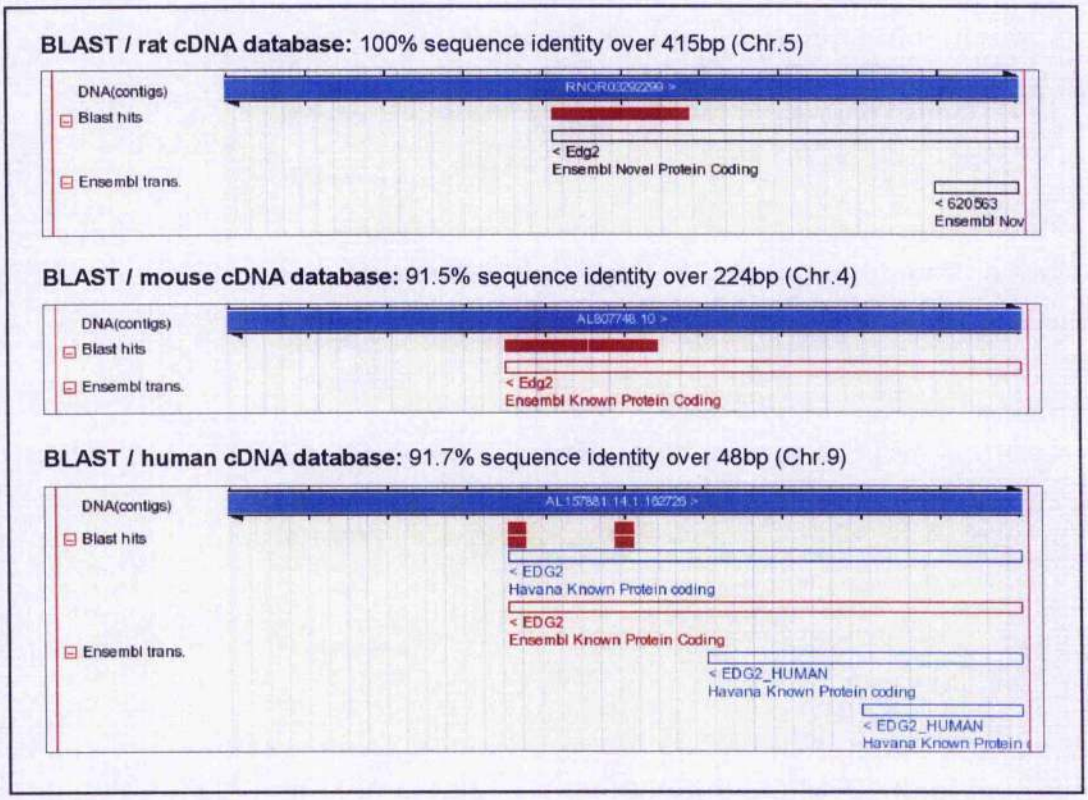
The initial aim of this project was to identify genes from the differentially expressed probe sets representing ESTs from the RG-U34B and RG-U34C GeneChip® data. A thorough DNA sequence homology screen strategy was used for identifying genes and ESTs from the Affymetrix probe set IDs. Data mining tools were used to determine their potential functional significance with relation to schizophrenia. On this basis, a small number of candidate genes were selected for confirmation by real-time PCR and *in situ* hybridisation.

3.2- Bioinformatics: homology screening to identify genes and ESTs

Lack of transcriptome information necessitated a DNA sequence homology screen in order to identify genes from 209 differentially expressed probe sets from the RG-U34B and RG-U34C GeneChip® data, representing ESTs. A comprehensive and thorough manual DNA sequence homology screen (against rat, mouse and human databases) was undertaken to identify a homologous gene for each differentially expressed rat probe set. Although this process was quite subjective, putative identified genes were ranked by decreasing confidence depending mostly on the degree of sequence identity of each probe set with different databases. Figures 3.1, A-C show representative examples of homology screens of three differentially expressed probe sets giving confident, uncertain and unsuccessful conversion into rat genes, respectively.

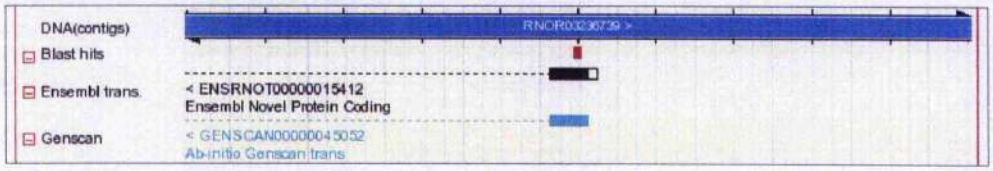
This rigorous analysis initially enabled the confident identification of 66 genes from the 209 differentially expressed EST probe sets (Appendix B) while extreme caution was preferred for the remaining majority of the ESTs (68% of the differentially expressed probe sets), considered as anonymous pieces of transcribed sequence.

A.

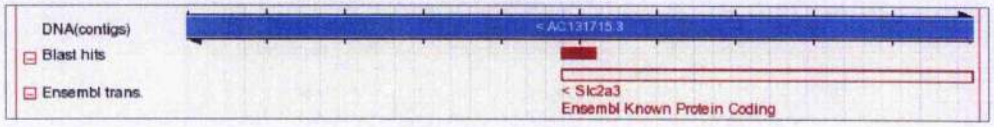


B.

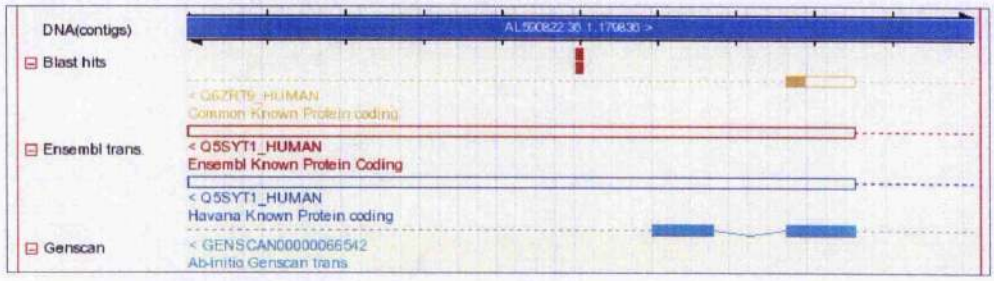
BLAST / rat cDNA database: 95.2% over 21bp (Chr.15)



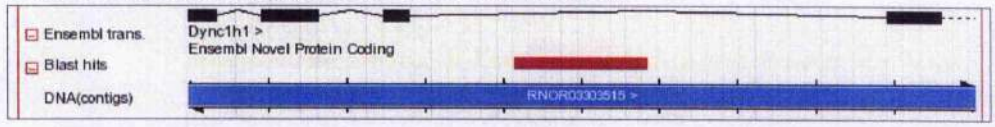
BLAST / mouse cDNA database: 95.7% over 94bp (Chr.6)



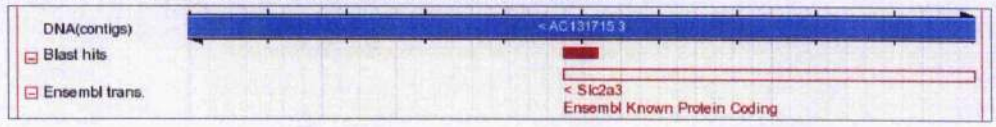
BLAST / human cDNA database: 100% over 20bp (Chr.1)



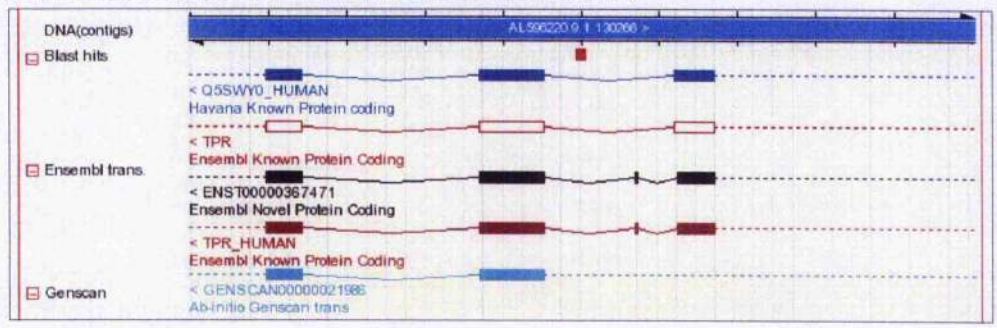
BLAST / rat genomic database: 100% over 412bp (Chr.6)



BLAST / mouse genomic database: 95.7% over 94bp (Chr.6)



BLAST / human genomic database: 96% over 25bp (Chr.1)



C.

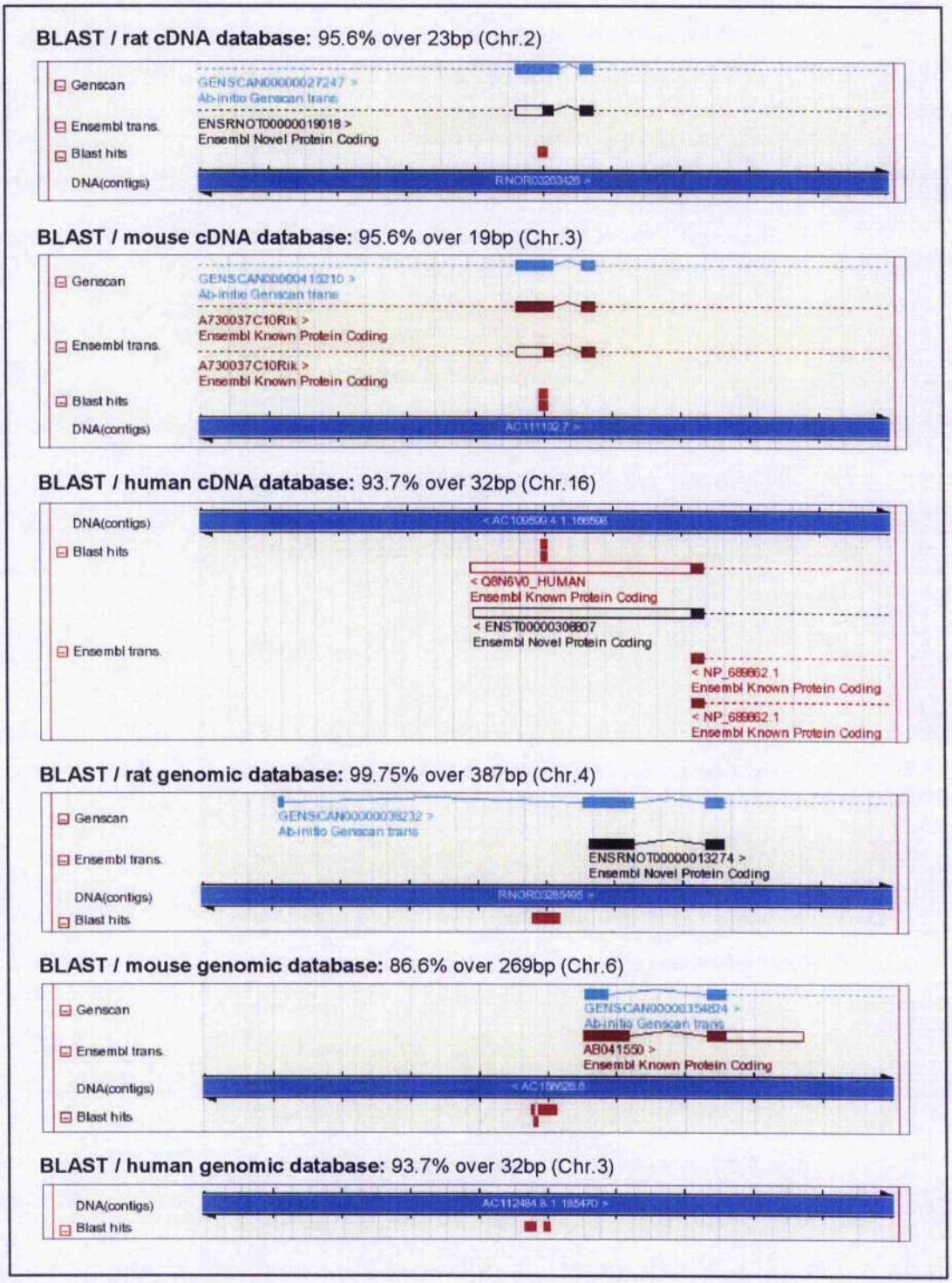


Figure 3.1, A-C. *Examples of homology screens allowing confident, uncertain and unsuccessful conversion into rat genes (respectively)*

In each case the consensus sequence retrieved from NetAffx™ website was aligned at ENSEMBL against rat, mouse and human cDNA and/or genomic databases. Figures give the results of these alignments in terms of length and percentage of sequence identity and show screenshots of ENSEMBL "contig views" showing the regions of the chromosomes around the BLAST hits.

In A, the consensus sequence was found to align well with rat, mouse and cDNA databases, identifying orthologues of the same rat gene, *Edg2*, across species.
In B, alignment of the consensus sequence with rat cDNA was insufficient to identify any rat gene, whereas good alignment with the rat genomic sequence favoured the identification of a gene called *Dync1h1*. However, this hypothesis was not confirmed by BLAST against mouse and human cDNA or genomic databases (neither mouse Slc2a3 nor any of the potentially identified human genes is an orthologue of *Dync1h1)*, casting doubt on the hypothesis that *Dync1h1* was the rat gene detected by this probe set.
In C, alignments of the consensus sequence against rat, mouse and human cDNA databases were all insufficient to identify any gene. The consensus sequence aligned reasonably with antisense rat and mouse genomic sequence located around potential transcripts; however, the genes identified in both species were not respective orthologues suggesting that the alignments were not genuine.

The homology screen was repeated as the databases are regularly updated and especially after the completion of the first draft of the rat genome sequence in February 2004. The most recent analysis identified 119 genes from the 209 differentially expressed probe sets thereby revealing additional genes that had remained anonymous in the initial analysis.

In summary, bioinformatics analysis of 209 differentially expressed probe sets resulted in the identification of 119 rat genes, rat genomic loci, human orthologous genes and their genomic loci (Appendix B).

3.3- Data mining for selecting candidate genes

Data mining analysis of the human genes, orthologues of the 119 differentially expressed rat genes was performed using open-access tools (Genecards®, Harvester) to retrieve genomic, transcriptomic and functional information potentially relevant for assessing the interest of investigating the genes in relation with schizophrenia. This information is shown in Appendix B.

The initial 66 differentially expressed genes were classified into different categories thereby providing a "snapshot" of the function of the genes and of the biological processes affected in the PCP model of schizophrenia (table 3.1).

Function	Number of genes
Transcription regulators	9
Signalling molecules	9
Cytoskeletal architecture	4
Receptors	4
Metabolism	4
Electron transport	3
Protein transport	2
RNA-binding	4
Mitochondrial	2
Antisense to a known gene	3
Unrelated function	22

Table 3.1. *Initial functional classification of 66 identified transcripts*

All retrieved information was also analysed in the context of schizophrenia to map the genes with well-supported regions linked to schizophrenia and investigate their functional relevance with schizophrenia itself and neuropsychopathology in general. Published gene expression (including microarray studies on human post-mortem tissue) data and previous association with schizophrenia were considered as well as fold changes of the differentially expressed ESTs in the microarray on the rat PCP model. Interestingly, it is worth mentioning here that 8/66 genes (*Fbn1, Sec63, Rac2, Ppp1r9b, Pik4cb, Hist1h4j, Q8wyq3, NM_016274* and *C10orf7*) were found to have human orthologues that are located at well-supported chromosomal regions linked to schizophrenia (Appendix B).

All this information together with the subjective appreciation of their suitability as drug targets, were used as criteria for selecting a small number of candidate genes to validate and characterise.

Two genes and one EST were selected as candidates for further validation and characterisation. *Edg2*, a G-protein coupled receptor that had been linked with schizophrenic-like behaviour (Harrison *et al.*, 2003) was selected as well as *Tm4sf12* (*tetraspanin 12*), a membrane protein potentially important in oligodendrocytes signalling. Interestingly, both of these genes presented relatively high fold changes (> 20%) compared to most significantly differentially expressed probe sets from this study, whose fold changes were less than ± 20%.

Following the same criteria and partly because it displayed the highest fold change among the RG-U34B and RG-U34C differentially expressed probe sets, one EST (rc_AI072720_at), which may or may not represent a genuine transcript, known or predicted gene, was selected for further validation and characterisation.

3.3.1- *Edg2*: data mining and reasons for selection

Edg2 was selected based on several factors, including its fold change in the rat microarray (>20% increase in the rat prefrontal cortex after chronic PCP treatment), the fact that it was a G-protein coupled receptor and therefore an obvious potential drug target and evidence showing that *Edg2* knock-out mice displayed schizophrenic-like behaviour (Harrison *et al.* 2003).

Edg2 (endothelial differentiation receptor 2), which is also called *LPA1* (lysophosphatidic acid receptor 1), is a G-protein coupled receptor for lysophosphatidic acid (Hecht *et al.* 1996; An *et al.* 1997), a lipid growth factor which causes various cellular events, including modulation of cell growth, differentiation, proliferation and migration as well as apoptotic and cytoskeletal effects (Handford *et al.* 2001; Yoshida and Ueda 2001). *Edg2* is widely expressed in human tissues with highest abundance in the brain (An *et al.* 1997) where its expression was shown to be temporally and spatially restricted, both during development and in the adult (Hecht *et al.* 1996). In particular, its embryonic expression profile suggests a potential role for *Edg2* in neurogenesis or neuroblast migration during development which is supported by *in vitro* studies showing Edg2-mediated morphological changes in neuroblasts (Contos *et al.* 2000a; Fukushima *et al.* 2000). Moreover, since *Edg2* expression reemerges in myelinating glia of the postnatal nervous system and follows the pattern of myelination in the early postnatal brain (Allard *et al.* 1998; Handford *et al.* 2001), it has been suggested to play a role in the control of myelination, a role which implicates this receptor in several psychiatric disorders (such as schizophrenia) suggested to have a developmental origin. In this context, *Edg2* knock-out mice were generated by two different groups who interestingly reported different phenotypes. Whereas Contos *et al.* (2000a) showed that these mice had deficits in suckling behaviour resulting in high neonatal lethality, Harrison *et al* (2003) did indeed show that *Edg2* receptor-deficient mice displayed a schizophrenic-like phenotype with deficits in prepulse inhibition, widespread changes in the levels

and turnover of the neurotransmitter 5-HT and alterations in levels of amino acids. Moreover, the targeted deletion of *Edg2* in the mice generated by Harrison *et al.* (2003) has since been shown to produce a number of changes in neurotransmitters (tyrosine, aspartate, glutamate, GABA) that have been associated with a schizophrenic-like pathology (Roberts *et al.* 2005).

In conclusion, the data generated by *Edg2* knock-out studies therefore provided further evidence that *Edg2* may be an interesting candidate (and maybe a potential drug target) to investigate in relation with schizophrenia.

3.3.2- *Tm4sf12:* data mining and reasons for selection

Interest in *Tm4sf12*, *tetraspanin 12*, mainly arose from its potential function and from its cellular localisation at the plasma membrane, which made it a potential target for designing new drugs. Moreover, *tetraspanin 12* had shown an increased expression of 20% in the rat prefrontal cortex after chronic PCP treatment, which was a notably high fold change by comparison with all the fold changes of differentially-expressed genes in this microarray. Like all members of the tetraspanin superfamily, tetraspanin 12 protein has four transmembrane domains delimiting two extracellular loops (Serru *et al.* 2000). However, contrary to others, it appears to be specifically expressed in the central nervous system where its mRNA is detectable shortly after birth and increases in level until the period of active myelination (Birling *et al.* 1999). Moreover, there is evidence of its specific expression in cells of the oligodendrocyte lineage which suggests that tetraspanin 12 may play a role in signalling in oligodendrocytes in the early stages of their terminal differentiation into myelin-forming glia (Bronstein 2000).

Tetraspanin 12 function and its altered expression in the chronic PCP model may therefore be related to white matter changes and evidence of altered myelination in schizophrenia (Davis *et al.* 2003; Kubicki *et al.* 2005; McInnes and Lauriat 2006). On this basis and because very little else was known about its function, it was selected as a candidate gene to validate and characterise in relation with schizophrenia.

3.3.3- EST AI072720: data mining and reasons for selection

The last candidate selected was the EST AI072720, which had shown the highest fold change of all differentially expressed RGU34B and C probe sets. Indeed, this EST had shown a 46% increase in expression in the rat prefrontal cortex after chronic PCP treatment, which was considered as a very large fold change since most changes were less than 20%. Moreover, although homology screening of the rc_AI072720_at probe set against genomic and cDNA databases had not allowed the definite identification of its homologous gene, the bioinformatics analyses had provided intriguing evidence that the EST AI072720 may correspond to a novel gene which was predicted to have a carboxy-terminal ezrin-radixin-moesin (ERM) domain. Since this domain is assumed to bind F-actin and in the light of the critical role of actin in cellular architecture, neuronal morphogenesis and synaptic plasticity (Luo 2002), such a gene may be related to the cytoskeletal alterations found in schizophrenia which have been proposed to represent the molecular origin of alterations in synaptic connectivity in this disorder (Harrison 1999; Benitez-King *et al.* 2004; Rapoport *et al.* 2005). Hence this gene could potentially represent a novel therapeutic target in schizophrenia (Benitez-King *et al.* 2004).
The EST AI072720 was thus selected on the basis of fold change, novelty and its potential function.

The data mining evidence used to select these 3 genes is summarised in table 3.2.

Gene	GeneChip % change	
Edg2 (lysophosphatidic acid G-protein-coupled receptor)	↗ 28% (RGU34C chip)	May play a role in modulating cell mobility. Knock-out mice show neurochemical and prepulse inhibition (PPI) deficits. Attractive drug target ?
Tm4sf12 (transmembrane 4 superfamily member tetraspan net-2)	↗ 20% (RGU34C chip)	Expression restricted to the CNS. May play a role in signalling in oligodendrocytes and may also function in stabilising myelin sheath.
EST AI072720	↗ 46% (RGU34C chip)	Homologous gene not definitively identified. Highest fold change from RG-U34B and RG-U34C differentially expressed probe sets. Putative protein may contain an ezrin, radixin, moesin domain. Potentially involved in cytoskeletal architecture.

Table 3.2. *Summary table of fold changes and information relevant to schizophrenia of the 3 selected candidate genes: Edg2, Tm4sf12 and EST AI072720*

3.4- Confirmation of differential expression of the candidate genes

Despite rigorous statistical analysis, microarray data cannot be exempt from false positives, and independent confirmation of the differential expression of candidate genes is essential before proceeding to undertake more elaborate characterisation experiments on them.

Quantitative real-time PCR (qRT-PCR) (section 2.4.5) and semi-quantitative *in situ* hybridisation (section 2.6), two methods that are commonly used for quantifying RNA, were used to this end.

For each gene, rat and human qRT-PCRs were performed, respectively in a set of cDNA samples prepared from the prefrontal cortex of rats that had been independently chronically treated with PCP and in cDNA samples prepared from post-mortem tissue (dorso-lateral prefrontal cortex) from schizophrenic patients and

controls (section 2.2.1). Results were analysed by ANCOVA using respective appropriate housekeeping genes as covariates (Bond *et al.* 2002).

In situ hybridisation was performed on brain sections from PCP-treated and vehicle-treated (controls) animals. Results were analysed using individual t-tests for each brain region to assess the differential expression of the genes after chronic PCP treatment.

3.4.1- *Edg2*

Using qRT-PCR, *Edg2* was found to show statistically significant differential expression after chronic PCP treatment in the rat prefrontal cortex. The qRT-PCR assays were repeated and the results were confirmed (1st time p=0.044, +19.3%, not shown ; 2nd time p=0.033, +29.7%, figure 3.2). Moreover, increased expression of *Edg2* after chronic PCP treatment was consistent with the microarray data.

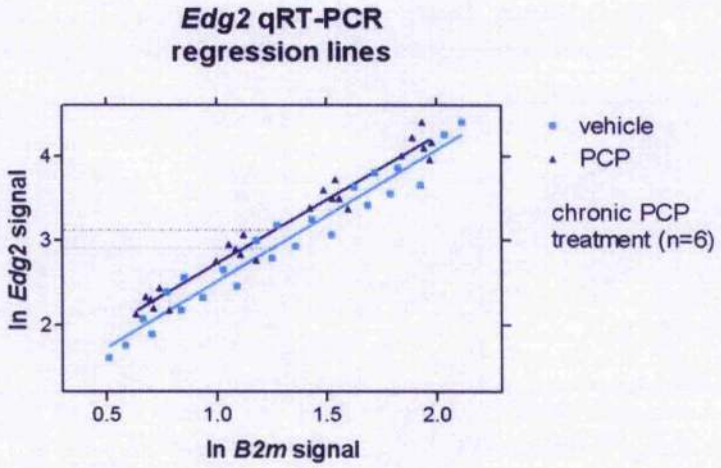


Figure 3.2. *Regression lines for rat Edg2 qRT-PCR*

qRT-PCR was performed to evaluate *Edg2* expression changes in the rat prefrontal cortex after chronic PCP treatment by comparison with a vehicle (saline solution) (n=6). This graph represents the repeat of this experiment which was performed twice. Y-axis is the logarithm of the fluorescence intensity representing the expression of *Edg2* and X-axis is the logarithm of the fluorescence intensity for *beta-2-microglobulin (B2m)*, the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which revealed 29.7% increase in *Edg2* expression after chronic PCP treatment (p=0.033). Dotted lines illustrate elevated ln *Edg2* signal intensities in PCP-treated rats for a particular ln *B2m* signal.

A human *EDG2* qRT-PCR assay was designed based on the rat Affymetrix target sequence to address the issue of isoform-specific differential expression and to specifically detect the isoform homologous to the rat *Edg2* transcript differentially expressed the rat microarray.

However no differential expression of human *EDG2* was detected in the entire data set (n=9, p=0.262, figure 3.3) or in the individual patient collections (Harvard, Brain-Net or UCLA; not shown).

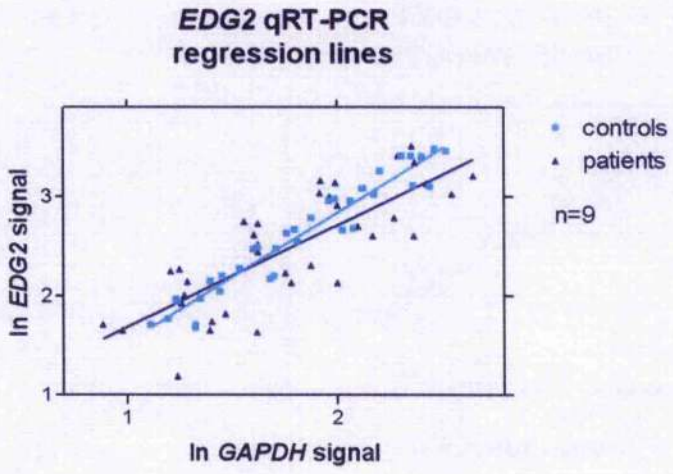


Figure 3.3. *Regression lines for human EDG2 qRT-PCR*

qRT-PCR was performed to evaluate *EDG2* expression changes in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients compared to controls (n=9). Y-axis is the logarithm of the fluorescence intensity representing the expression of *EDG2* and X-axis is the logarithm of the fluorescence intensity for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which did not reveal any significant change in *EDG2* expression in schizophrenic patients (p=0.262).

In situ hybridisation was employed to investigate the regional expression profile of *Edg2* mRNA in the rat brain and its potential alteration in response to chronic PCP administration (figure 3.4).

Edg2 mRNA was expressed in several brain regions that have been implicated in schizophrenic pathology, especially the prefrontal cortex (A), the thalamus and the hippocampus (B). *Edg2* mRNA expression was highest within white matter tracts throughout the brain. The hippocampus displayed the highest mRNA levels within grey matter, with lower and homogeneous cortical and subcortical expression of *Edg2* mRNA detected.

28 regions of the rat brain were analysed for differential expression of *Edg2* expression : prelimbic cortex (PrL), infralimbic cortex (iL), ventral orbital cortex (vO), lateral orbital cortex (lO), primary and secondary motor cortices (M1 and M2), anterior cingulate cortex (acg), granular (rsg) and agranular (rsa) retrosplenial cortices, dentate gyrus granule cell layer (DGgcl), pyramidal cell layers of the CA1 (CA1pcl), CA2 (CA2pcl) and CA3 (CA3pcl) region of the hippocampus, primary auditory cortex (Au1), lateral entorhinal cortex (Lent), corpus callosum (cc), genu of corpsu callosum (gcc), forceps minor (fmi) and forceps major (fmj) corpus callosum, anterior commisure (ac), intrabulbar part of the anterior commisure (aci), external (ec) and internal (ic) capsules, cingulum (cg), stria medullaris of the thalamus (sm), mammillothalamic tract (mt), fornix (f) and fimbria of hippocampus (fi).

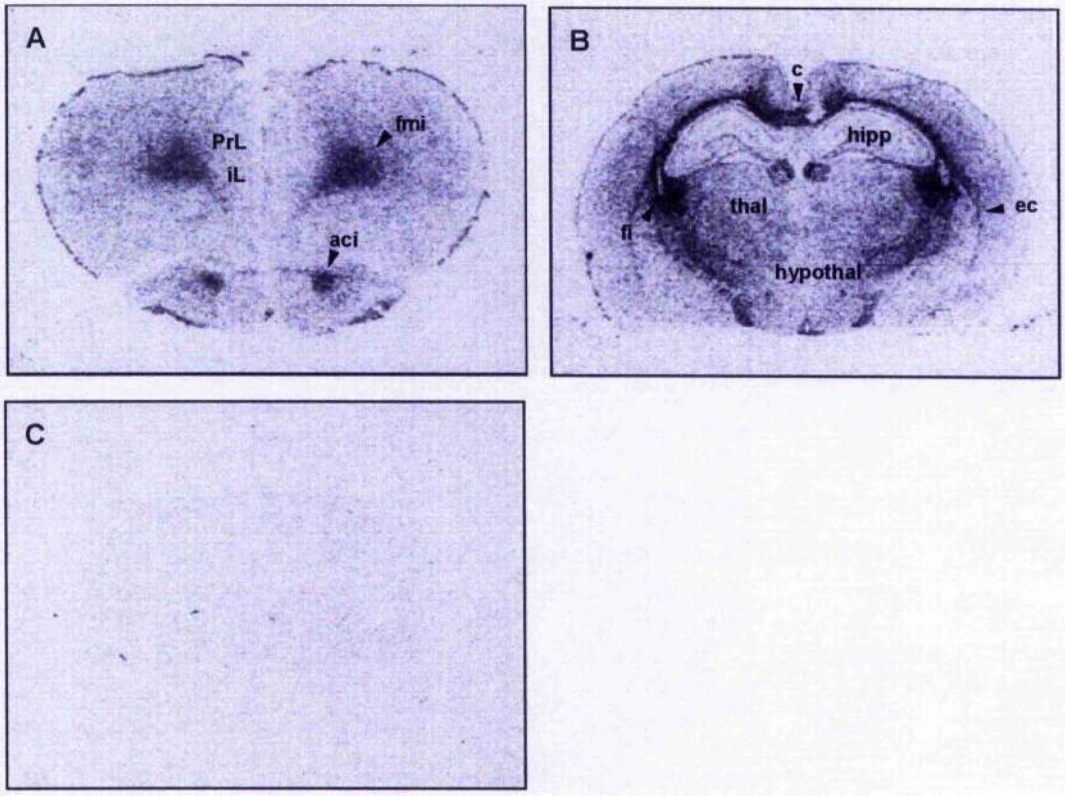


Figure 3.4. *Representative sections illustrating the regional distribution of Edg2 mRNA in selected regions of the rat brain (panels A and B) and control section (panel C)*

Coronal sections used for *Edg2 in situ* hybridisation experiments were obtained from the following bregma levels according to Paxinos (1998): A. 3.20mm (prefrontal cortex), B. -2.30mm (midline thalamus) (Paxinos 1998). Sections were hybridised with ^{35}S-labelled oligonucleotide probes specific for *Edg2* (in presence of an excess of unlabelled probe in panel C), exposed to film for 17 days and digitally scanned. Dark areas mark the presence of mRNA.

PrL: prelimbic cortex; iL: infralimbic cortex; fmi: forceps minor corpus callosum; aci: intrabulbar part of the anterior commisure: cc: corpus callosum; ec: external capsule; fi: fimbria of hippocampus; hipp: hippocampus; thal: thalamus; hypothal: hypothalamus.

The uniform, non-specific signal shown in panel C) provides confirmation of the specificity of the probes used for *Edg2 in situ* hybridisation studies.

In situ hybridisation did not show any significant effect of PCP treatment on *Edg2* expression in prefrontal cortical areas unlike the differential expression detected by the microarray and by qRT-PCR (figure 3.5) Moreover, there were no significant effects of PCP treatment on *Edg2* expression in all the regions of the white matter analysed (figure 3.5).

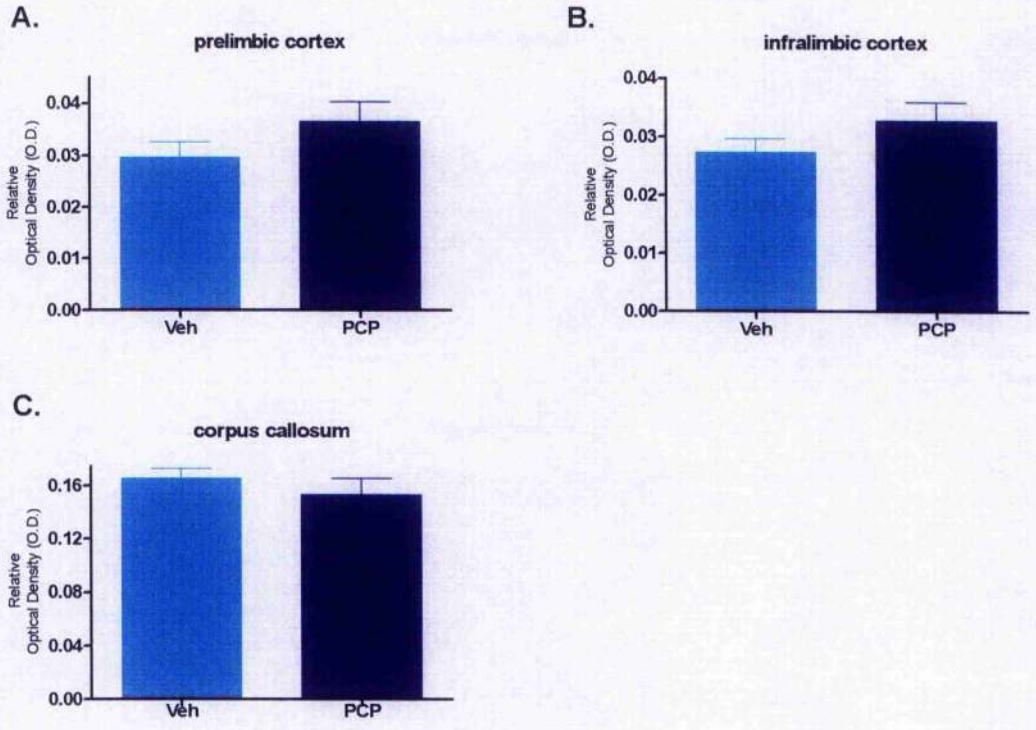


Figure 3.5. *Bar graphs for rat Edg2 in situ hybridisation*

Results shown are mean ± standard error of the mean (s.e.m.)
n=6 animals for vehicle and PCP-treated rats
A. prelimbic cortex; B. infralimbic cortex; C. corpus callosum

Edg2 mRNA showed a heterogeneous expression profile throughout the rat brain. High levels of *Edg2* were detected in the white matter consistent with published literature (Hecht *et al.* 1996; Allard *et al.* 1998; Handford *et al.* 2001). However, *Edg2* appears to also be expressed in the cortex as well as in the thalamus and hippocampus, key regions implicated in schizophrenic pathology.

No significant changes in *Edg2* expression were found in either the prefrontal cortex or in the white matter following chronic PCP treatment.

3.4.2- *Tm4sf12*

Differential expression of *Tm4sf12* was not observed in the prefrontal cortex of rats treated chronically with PCP when tested by qRT-PCR (figure 3.6).

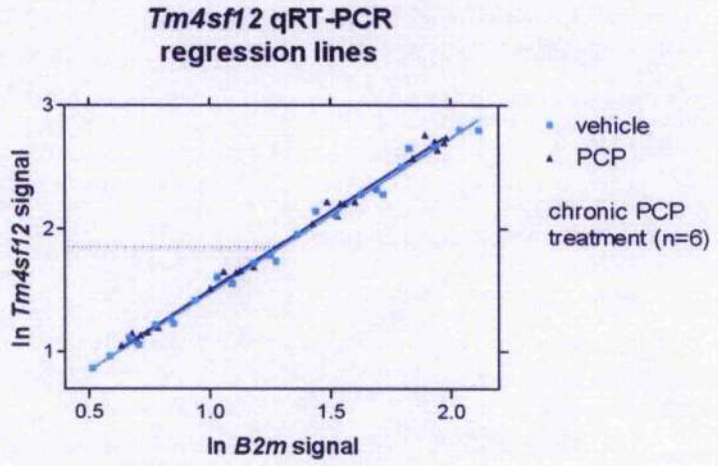


Figure 3.6. *Regression lines for rat Tm4sf12 qRT-PCR*

qRT-PCR was performed to evaluate *Tm4sf12* expression changes in the rat prefrontal cortex after chronic PCP treatment by comparison with a vehicle (saline solution) (n=6). This graph represents the first of two experiments. Y-axis is the logarithm of the fluorescence intensity representing the expression of *Tm4sf12* and X-axis is the logarithm of the fluorescence intensity for *beta-2-microglobulin (B2m)*, the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which did not reveal any significant change in *Tm4sf12* expression after chronic PCP treatment (p=0.303). Dotted lines illustrate unchanged ln *Tm4sf12* signal intensities in PCP-treated rats for a particular ln *B2m* signal.

qRT-PCR was also performed on human *TM4SF12* using post-mortem dorso-lateral prefrontal cortex from schizophrenic patients and controls but no differential expression was observed, analogous to the rat prefrontal cortex (figure 3.7).

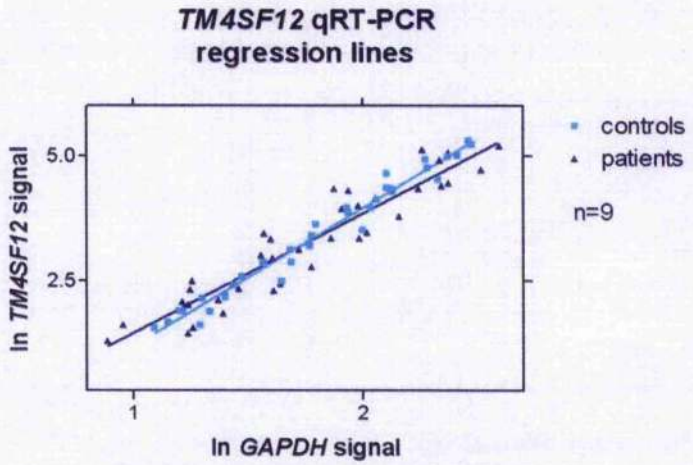


Figure 3.7. *Regression lines for human TM4SF12 qRT-PCR*

qRT-PCR was performed to evaluate *TM4SF12* expression changes in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients compared to controls (n=9). Y-axis is the logarithm of the fluorescence intensity representing the expression of *TM4SF12* and X-axis is the logarithm of the fluorescence intensity for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which did not reveal any significant change in *TM4SF12* expression in schizophrenic patients (p=0.449).

All qRT-PCR results were therefore consistent suggesting that *Tm4sf12* differential expression in the chronic PCP model (revealed in the microarray) was not genuine or was too small to be reliably independently confirmed by other techniques.

Consequently, *in situ* hybridisation, which is a semi-quantitative method less powerful than qRT-PCR for identifying gene expression changes, was not performed on this gene.

3.4.3- EST AI072720

Using qRT-PCR, the EST AI072720 was confirmed as differentially expressed in the rat prefrontal cortex after chronic PCP treatment. The rat qRT-PCR assay was performed on an independent set of rat prefrontal cortex samples and showed a significant upregulation ($p<0.05$, +16.5%) of the EST AI072720 after chronic PCP treatment (figure 3.8) which was consistent with the microarray data.

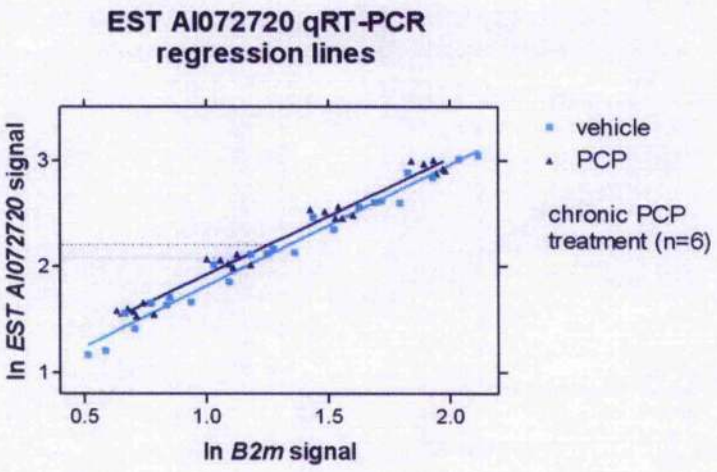


Figure 3.8. *Regression lines for rat EST AI072720 qRT-PCR*

qRT-PCR was performed to evaluate EST AI072720 expression changes in the rat prefrontal cortex after chronic PCP treatment by comparison with a vehicle (saline solution) (n=6). This graph represents the repeat of this experiment which was performed twice. Y-axis is the logarithm of the fluorescence intensity representing the expression of the EST AI072720 and X-axis is the logarithm of the fluorescence intensity for *beta-2-microglobulin (B2m)*, the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which revealed 16.5% increase in the expression of the EST AI072720 after chronic PCP treatment (p=0.04). Dotted lines illustrate elevated ln *EST AI072720* signal intensities in PCP-treated rats for a particular ln *B2m* signal.

A human qRT-PCR assay was designed based on the rat Affymetrix target sequence to detect the human orthologous region of rat EST AI072720 which was found to correspond to the 3' untranslated region (3'UTR) of a predicted gene called *KIA1189* (section 5.2).

No differential expression of *KIAA1189* expression was observed in human post-mortem DLPFC from schizophrenic patients, neither in the entire set of patients and controls (n=9, p=0.888, figure 3.9) nor in the individual collections of patients (Harvard, Brain-Net or UCLA, data not shown).

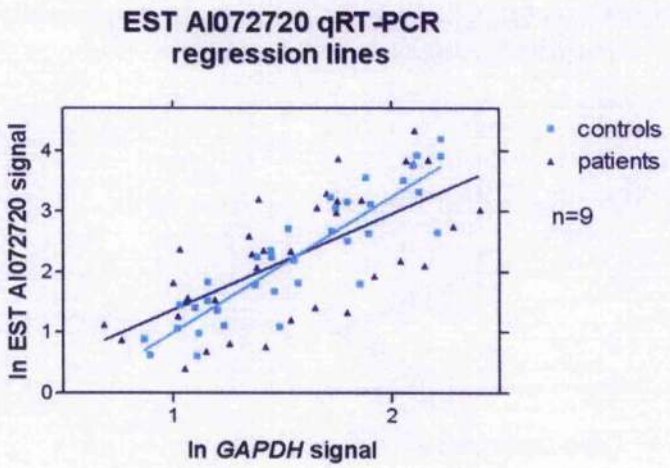


Figure 3.9. *Regression lines for human EST AI072720 qRT-PCR*

qRT-PCR was performed to evaluate EST AI072720 expression changes in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients compared to controls (n=9). Y-axis is the logarithm of the fluorescence intensity representing the expression of the EST AI072720 and X-axis is the logarithm of the fluorescence intensity for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which did not reveal any significant change in EST AI07270 expression in schizophrenic patients (p=0.888).

In situ hybridisation was employed to investigate the regional expression profile of EST AI072720 mRNA in the rat brain and its potential alteration in response to chronic PCP administration (figure 3.10).

This EST was found to be highly expressed within all major white matter tracts. Within cortical and subcortical areas, it was expressed at a much lower level and had a homogeneous distribution. Expression was observed within all cortical areas, the striatum, thalamus, hypothalamus and hippocampus, with the prefrontal cortex appearing to display the lowest level.

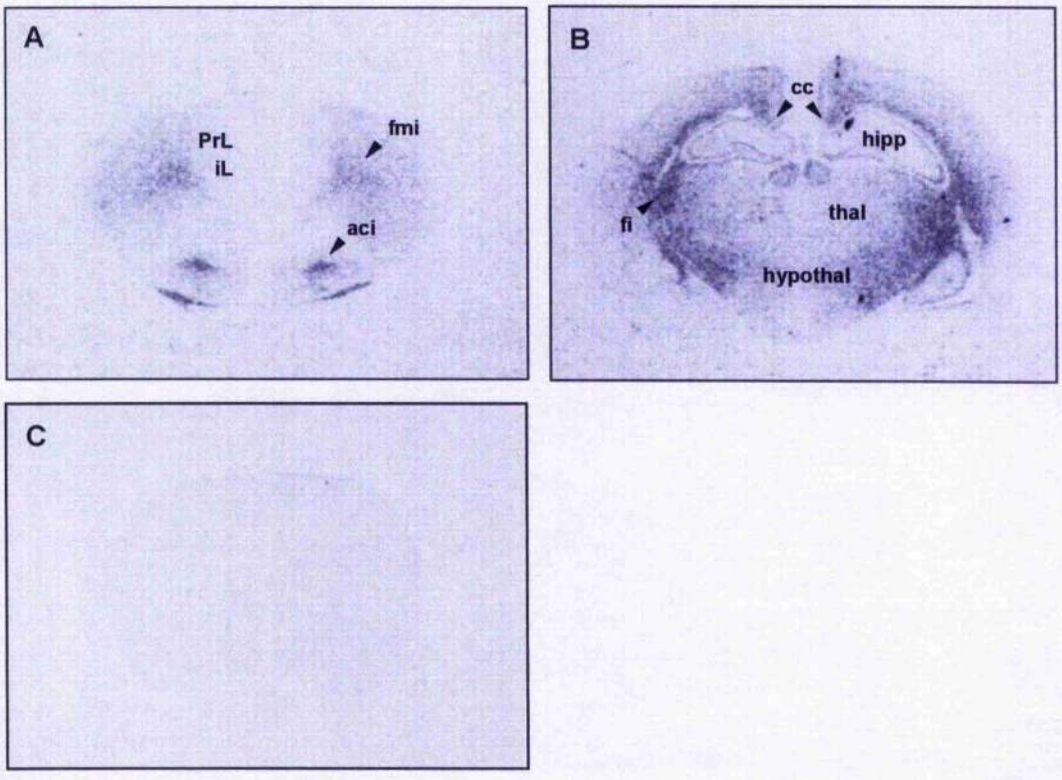


Figure 3.10. *Representative sections illustrating the regional distribution of EST AI072720 mRNA in selected regions of the rat brain (panels A and B) and control section (panel C)*

Coronal sections used for EST AI072720 *in situ* hybridisation experiments were obtained from the following bregma levels according to Paxinos (1998): A) 3.20mm (prefrontal cortex), B) -2.30mm (midline thalamus) (Paxinos 1998). Sections were hybridised with ^{35}S-labelled oligonucelotide probes specific for the EST AI072720 (in presence of an excess of unlabelled probe in panel C), exposed to film for 1 month and digitally scanned. Black areas mark the presence of mRNA.
PrL: prelimbic cortex; iL: infralimbic cortex; fmi: forceps minor corpus callosum; aci: intrabulbar part of the anterior commisure: cc: corpus callosum; fi: fimbria of hippocampus; hipp: hippocampus; thal: thalamus; hypothal: hypothalamus.
The uniform, non-specific signal shown in panel C) provides confirmation of the specificity of the probes used for EST AI072720 *in situ* hybridisation studies.

16 regions of the rat brain were analysed for differential expression of EST AI072721 expression: corpus callosum (cc), genu of corpus callosum (gcc), forceps minor (fmi) and forceps major (fmj) corpus callosum, anterior commisure (ac), intrabulbar part of the anterior commisure (aci), external capsule (ec), cingulum (cg), stria medullaris of the thalamus (sm), fimbria of hippocampus (fi), anterior cingulate cortex (acg), granular (rsg) and agranular (rsa) retrosplenial cortex, pyramidal cell layers of the CA1 (CA1pcl), CA2 (CA2pcl) and CA3 (CA3pcl) region of the hippocampus.

No significant change was found in the expression levels of EST AI072720 in any of the regions after chronic PCP treatment. However, there was a trend towards increased expression of the EST AI072720 in the CA3 pyramidal cell layer of the hippocampus after chronic PCP treatment (figure 3.11).

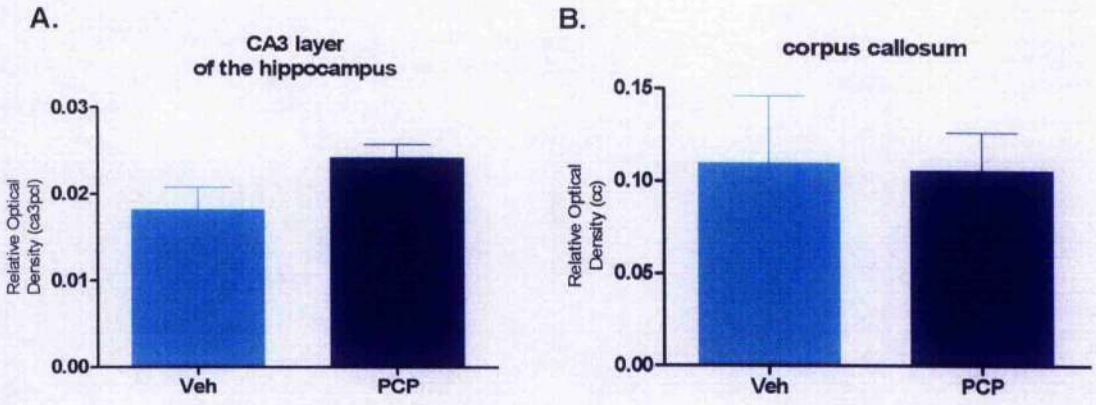


Figure 3.11. *Bar graphs for rat EST AI072720 in situ hybridisation*

Results shown are mean ± standard error of the mean (s.e.m.)
n=6 animals for vehicle and PCP-treated rats
A. CA3 layer of the hippocampus; B. corpus callosum.

3.5- Discussion

The aim of the work described in this chapter was to select a few candidates from the genes identified in the rat microarray and to confirm their differential expression after chronic PCP treatment by two independent techniques, qRT-PCR and semi-quantitative *in situ* hybridisation.

3.5.1- Identification of differentially-expressed genes (DNA homology screening)

Thorough bioinformatic analyses of the 209 differentially expressed probe sets from the RG-U34B and RG-U34C GeneChip® microarray data of prefrontal cortex isolated from rats treated chronically with PCP were conducted to identify differentially expressed genes: from the ESTs, only 66 homologous genes were identified whilst 143 therefore remained ESTs. By comparison, 104 out of the 118 probe sets differentially expressed in the RG-U34® GeneChip data were converted into genes using the same process (Catherine Winchester) which reflects the complexity of interpretating data on ESTs rather than from characterised full-length sequences of better sequence quality.

Failure to identify genes for such a large proportion of the probe sets may be due to the paucity of available annotation of the rat transcriptome (especially on untranslated regions) and to differences in the annotation level of databases of different species (rat, mouse and human) which often resulted in poor or contradictory alignments (including strand orientation issues). Moreover, even though ESTs are a precious tool for genome annotation, the overall quality of any individual sequence within all collections of ESTs remains one of the major problems which affects homology screening with genomic sequences and therefore complicates gene identification (Rudd 2003). Assigning ESTs to genes based on DNA sequence homology screens is subjective. In this thesis the conversion was rigorous and cautious, with cut off points of above 90% sequence identity for the rat alignments and less demanding requirements for mouse (about 80%) and human (about 70%) alignments according to the distance separating these species from the rat on the phylogenetic tree. This was to ensure confidence in the genes identified. It is therefore possible that more ESTs could have been converted if less rigorous

decisions were made. Instead, a number of alignments were considered as insufficiently good for confidently establishing the corresponding EST and transcript. Moreover, ESTs could have been assigned to gene families, even if the corresponding gene was not identified.

However, even if it can be assumed that a number of unassigned ESTs are therefore likely to correspond to genuine transcripts, not all of them do and there are reasons to explain the origin of these non-coding ESTs. First, contamination of EST sequence collections by genomic DNA or cDNA from other species ("xenocontaminants") appear to be an almost unavoidable problem since they are still hardly detectable despite improvements in the quality control of EST sequences that are being deposited in databases (Jongeneel 2000). EST databases may also contain "mosaic ESTs" arising from multiple ligations during cDNA library construction or structural or regulatory RNAs that should not have been cloned hence sequenced (for example, internal priming on intronic A-rich sequences in nuclear hnRNA is quite common) (Rudd 2003).

In theory, EST databases should not include microRNA (miRNA), which do not have a poly(A)tail and therefore should have been lost during cloning (assuming that oligo-dTs were used to prime first strand synthesis). However, since miRNA are usually expressed as part of longer transcripts and do not necessarily reside in introns, they may be represented in EST databases as chimeric transcripts encoding both a microRNA precursor and a region of an adjacent mRNA (Smalheiser 2003). Moreover, EST analysis has been shown to be very useful for identifying new plant miRNAs (Zhang *et al.* 2005b) suggesting that EST databases actually include a number of miRNAs. Some ESTs which were not translated into genes may therefore represent miRNAs which may play a crucial role in the regulation of gene expression by chronic PCP treatment.

Finally, extensive bioinformatics analyses would be necessary to examine each of the unassigned ESTs in detail and to determine to what it corresponds. Such studies may be particularly interesting as they may have the power to identify not only novel genes but also novel miRNAs which have recently emerged as potent regulators of gene expression during development (Bartel 2004; Klein *et al.* 2005).

3.5.2- Selection of candidate genes (data mining)

Data mining was performed on the 66 differentially expressed genes in order to assess their potential relevance to schizophrenia and to select a few candidates to validate. A number of criteria were used to this aim, including previous linkage and/or association with schizophrenia and known or putative gene/protein function. Fold changes of probe sets in the microarray were also noted; however since detection of gene expression changes using brain tissue can be affected by many factors, fold changes may not necessarily reflect biological significance (Mirnics and Pevsner 2004). Thus, it is worth mentioning that the modest magnitude of expression changes found in this microarray (the large majority of fold changes were below 20% increased or decreased expression) was expected partly because of the potential phenotypic diversity of the samples and of the limited regulation of transcription in the mature brain (Mirnics and Pevsner 2004). Fold changes were therefore not strictly considered a criterion for this selection, which mostly remained a subjective and arbitrary process. Two genes (*Tm4sf12* and *Edg2*) and one EST (AI072720) were selected but others may have been very interesting as well.

Among these genes is *filamin A*, an X-linked gene which links actin cytoskeleton to various transmembrane proteins and receptors (including dopamine receptors; Lin *et al.* 2001) and serves as a scaffold for a wide range of cytoplasmic signalling proteins (Robertson 2004). Filamin plays a critical role in a particular stage of neuronal migration and was shown to be disrupted in several disorders of neocortical development (LoTurco and Bai 2006) which may be consistent with the neurodevelopmental hypothesis of schizophrenia.

Another gene whose implication with schizophrenia could be related with this hypothesis is the *ephrin type-A receptor 2 precursor* (*Epha2*), a receptor expressed in projecting neurons and their target fields, and involved in axonal pathfinding (Orioli and Klein 1997).

Mu-protocadherin isoform 3 (*Mucdhl*) may also have been an interesting candidate as this cell-surface adhesion molecule is involved in the development and maintenance of neural circuitry and therefore may have underlined abnormalities in synaptic connectivity and plasticity shown in schizophrenia. However, since a few studies on different members of this family failed to confirm their role in the

susceptibility to schizophrenia or other psychiatric disorders (Bray *et al.* 2002; Kirov *et al.* 2003; Giouzeli *et al.* 2004; Durand *et al.* 2006), this gene was not selected.

Myocyte-specific enhancer factor 2C (*Mef2c*) is one of the genes which displayed a notably high fold change in the microarray, with a decrease in expression of 34% in the rat prefrontal cortex after chronic PCP treatment. This transcription factor notably implicated in activity-dependent neurogenesis (Okamoto *et al.* 2002) has been suggested to mediate infrastructure changes associated with bipolar disorder (Ogden *et al.* 2004) in a mechanism which may implicate another gene identified in our microarray, the *proenkephalin A precursor* (*Penk*) (Ogden *et al.* 2004). Since schizophrenia and bipolar disorder are known to have common basic neurobiological processes (Maier *et al.* 2006), both of these genes may have been selected for further validation and investigation in the context of schizophrenia.

Other genes whose function may be related to schizophrenia pathophysiology include:

Slc7a10, a member of the solute carrier family which may play a role in the modulation of glutamatergic transmission through mobilisation of D-serine at the glutamatergic synapse (Nakauchi *et al.* 2000) and therefore may directly be related to NMDA receptor function in schizophrenia,

Fbn1 (*fibrillin 1*), a gene whose locus, chromosome 15q15, is linked with schizophrenia and whose mutations cause Marfan syndrome, a connective tissue disorder which segregates with schizophrenia (Kalsi *et al.* 1994),

Pik4cb (*phosphatidylinositol 4-kinase beta polypeptide*), a gene located in chromosome 1q21.3, one of the strongest loci linked with schizophrenia and involved in various signalling pathways potentially altered in schizophrenia (Stopkova *et al.* 2004). Interestingly, along with other members of its family, *PIK4CA*, the gene encoding the other polypeptide of the kinase, is also located in a key schizophrenia locus (22q11) and has been investigated in schizophrenia (Saito *et al.* 2003),

Hist1h4j (*histone 1, H4j*), a histone gene located in one of the strongest schizophrenia loci as well (chromosome 6p22.1) whose modification may contribute to the pathogenesis of prefrontal dysfunction in schizophrenia, especially through methylation at gene promoters (Akbarian *et al.* 2005),

Vamp5 (*vesicle-associated membrane protein 5*), a presynaptic terminal protein which may be involved in synaptic plasticity (Halim *et al.* 2003) and

Myelin-basic protein (*Mbp*), which encodes the major and specific constituent of myelin and is the only gene from all identified differentially-expressed genes which had already been studied in schizophrenia. Altered expression of *Mbp* in the rat prefrontal cortex after chronic PCP treatment directly relates to impaired myelination in schizophrenia (Hakak *et al.* 2001; Pongrac *et al.* 2002; Davis *et al.* 2003; Lehrmann *et al.* 2003; Tkachev *et al.* 2003), which is probably the most consistent finding among gene expression studies of post-mortem brain tissue from subjects with schizophrenia (Davis *et al.* 2003; Kubicki *et al.* 2005; McInnes and Lauriat 2006).

3.5.3- Validation of candidate genes – qRT-PCR

As microarray studies are known to be error-prone (Chuaqui *et al.* 2002), validating their results was considered a prerequisite before undertaking any further characterisation experiments on selected candidate genes. In order to provide not only a technical but also a biologically meaningful confirmation of these results, an independent set of cDNA samples from rats treated chronically with PCP or vehicle were used for qRT-PCR.

Using qRT-PCR, two of the three selected candidates, *Edg2* and the EST AI072720, were confirmed to be differentially expressed in the rat prefrontal cortex after chronic PCP treatment using the independent set of samples. Interestingly, both genes showed increased expression after chronic PCP treatment which was consistent with the microarray. qRT-PCR fold changes were smaller than the microarray ones which may be unexpected since qRT-PCR is usually more sensitive than microarray detection (Yuen *et al.* 2002b; Czechowski *et al.* 2004; Dallas *et al.* 2005). However, since fold changes are related to many parameters (including probe length and properties, absolute abundance of transcript, data standardisation) it would be inappropriate to directly compare microarray and qRT-PCR fold changes. In addition, consistency in the direction of the change was considered as further support of the potential implication of these genes in schizophrenia.

Differential expression of *EDG2* and the EST AI072720 was not confirmed by qRT-PCR in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients and therefore did not provide further support of the potential involvement of these genes in schizophrenia. Nevertheless, the number of issues that are inherent to both the methodology and the design of this study strongly suggested that these results should be considered as a lack of evidence rather than negative results. Thus, despite the good validity of the rat chronic PCP model (section 1.1.4.4), this study was compounded by the difficulty in confirming small expression changes detected in microarrays and by the complexity of any gene expression analysis of brain tissue. In addition, potential isoform differences between species cannot be excluded, and the correspondence between gene expression changes in a particular brain region of a particular animal model of a disease and gene expression changes in the homologous brain region of actual people diagnosed with this disease is likely not to be direct. Moreover, schizophrenia, like all psychiatric disorders, is far from being uniform and the clinical heterogeneity between patients (Lewis and Levitt 2002) is likely to reflect differences in pathophysiology and in gene expression patterns. This heterogeneity further adds to differences in genetic background and natural gene expression profiles across populations (both within and across the examined groups) which may also mask gene expression changes between different collections of people (Mirnics and Pevsner 2004). Finally, failure to translate gene expression changes found in the rat prefrontal cortex after chronic PCP treatment into gene expression changes in human port-mortem dorso-lateral prefrontal cortex from schizophrenic patients may be explained by the problem of modelling such a complex disease as schizophrenia in an animal and the homology between the examined brain regions. Thus, the rat chronic PCP model has been shown to mimic many pathophysiological features of schizophrenia but has particularly good validity towards the cognitive deficits of schizophrenia (section 1.1.4.4). However, no information was available as to the cognitive impairments exhibited by the schizophrenic patients used for the qRT-PCR analysis, so that the rat chronic PCP model may show gene expression changes potentially relevant to schizophrenia but not shown by this specific collection of schizophrenic patients. Moreover, it is very difficult when working across species to ascertain exact homology between brain regions. In particular, the prefrontal cortex has been found to show enormous variation across species in terms of anatomical criteria such as cytoarchitectonics and

connectivity (Dalley *et al.* 2004). In the way the rat prefrontal cortex was dissected both for the microarray study and the qRT-PCR analyses, it included the prelimbic cortex, the infralimbic cortex and parts of the ventral orbital and motor (M2) cortices. Because direct comparison with the human brain on purely anatomical grounds is not straightforward (Kolb 1984), trying to find the exact homologues of these regions in the human brain would have been impossible, especially since the availability of post-mortem material from schizophrenic patients was also very limiting. Human qRT-PCR analyses were therefore performed on post-mortem Brodmann area 10 from schizophrenic patients and controls. Strictly speaking, this region, known as the frontal pole or rostral frontal cortex, represents the anterior prefrontal cortex of the human brain and may be one of its least well understood regions (Ramnani and Owen 2004). However, this region is commonly referred to as part of the dorso-lateral prefrontal cortex, which usually includes a combination of regions, namely Brodmann areas 9, 10, 44, 45 and 46 and has been used in many studies on schizophrenia (Kawasaki *et al.* 2000; Vogeley *et al.* 2003; Katsel *et al.* 2005). Moreover, the human dorso-lateral prefrontal cortex as a whole has been suggested to represent the functional homolog of the rat prelimbic cortex (Wilson *et al.* 2004), *i.e.* a portion of our dissected rat brain samples. Our rat and human samples were therefore grossly functionally homologous but were also likely to present substantial differences in cellular phenotypes and connectivity pattern (Uylings and van Eden 1990; Gabbott *et al.* 1997), which may also explain the fact that gene expression changes found in the rat chronic PCP model were not confirmed in schizophrenic patients.

Very interestingly, evidence supporting the hypothesis that these issues may explain the failure to confirm differential expression of *EDG2* in human post-mortem dorso-lateral prefrontal cortex was provided by gene expression data available in the BioExpress® Central Nervous System (CNS) Suite (GeneLogic, USA) (obtained through Mitsubishi Pharma Corporation, Japan). In addition to revealing that *EDG2* expression was significantly higher in the CNS than in other tissues, this information from Affymetrix GeneChip® microarray studies showed indeed that *EDG2* mRNA expression was significantly increased in schizophrenic patients (n=24) compared to controls (n=40) in the entorhinal cortex (Brodmann area 36) and in the anterior prefrontal cortex (Brodmann area 10), the latter being the same region as the one our human post-mortem samples came from. These data therefore provided evidence that

EDG2 may be involved in schizophrenia, representing a concrete example of the potential importance of the previously discussed issues and thereby further favouring an extreme cautiousness in the analysis of gene expression data particularly those obtained using post-mortem tissue.

Contrary to the two other candidates, *tetraspanin 12* was not confirmed to be differentially expressed, neither in the rat prefrontal cortex after chronic PCP treatment (in an independent set of samples or in the microarray samples) nor in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients. Failure to confirm differential expression of this gene may not be unexpected though since its 20% increased expression after chronic PCP treatment (notably lower than the 46% increased expression of the EST AI072720 in the same conditions) was considered a very low fold change in the microarray field, difficult to confirm by qRT-PCR (Dallas *et al.* 2005). Thus, these results were consistent with evidence showing a trend towards poorer correlation of microarray and qRT-PCR results for genes exhibiting fold changes less than 1.5 (*i.e.* $<\pm 50\%$ expression) compared to those with fold changes greater than 1.5 (*i.e.* $>\pm 50\%$ expression) (Dallas *et al.* 2005). It has even been shown that validation is not consistently achieved for genes showing fold changes less than 4 (*i.e.* $+300\%$ expression) (Rajeevan *et al.* 2001) which, in the context of the success in confirming confirming expression changes of other genes, especially *Edg2* (which showed 28% increase in expression in the microarray), rather suggested that our qRT-PCR methodology and practise had a very high potential. Thus, failure to confirm the differential upregulation of *tetraspanin 12* after chronic PCP treatment, by comparison with *Edg2*, suggested that its differential expression may not be genuine. qRT-PCR analysis of *tetraspanin 12* expression in human post-mortem brain tissue from schizophrenic patients did not refute this hypothesis since it also failed to confirm any expression changes, and thereby provide evidence that this gene may be involved in schizophrenia. Therefore, although its function may have fitted very well with current hypotheses on schizophrenia, *tetraspanin 12* was suggested to represent a false-positive which had been identified in the rat microarray along with differentially expressed genes, and it was therefore decided not to pursue any more work on this gene.

3.5.4- Validation of candidate genes – *in situ* hybridisation

The purpose of using *in situ* hybridisation as part of the validation of the candidate genes was two-fold. First, it was used to determine the expression pattern of the candidates within the brain thereby providing potential insight into their function. Second, as an independent anatomical verification strategy, it was used in a semi-quantitative manner to confirm expression changes of candidate genes in the rat prefrontal cortex and evaluate potential changes in other brain areas outside the prefrontal cortex.

Surprisingly, *Edg2* and the EST AI072720 were found to exhibit very similar expression patterns throughout the brain, with highest expression within all white matter tracts and low expression levels within cortical areas. However, within grey matter, both mRNAs were expressed in several brain regions that have been implicated in schizophrenic pathology, including the prefrontal cortex and the thalamus and hippocampus, which probably displayed the highest mRNA levels within grey matter for both genes.

Such a similarity between expression patterns of the two examined genes may cause concern and raise the issue of the specificity of the probes. However every probe was tested and checked for specificity prior to hybridisation to the proper experimental slides (section 2.6). Therefore the similarity (but not exact identity) between the expression patterns of *Edg2* and the EST AI072720 appeared to be coincidental and both genes were genuinely highly expressed within the white matter by comparison with cortical areas. In addition, the hybridisation signal with each probe was completely absent when an excess of the same unlabelled probe was added, suggesting specificity of hybridisation.

The white matter, which plays a major role in connecting different brain regions together, is comprised of axonal projections of neurons located in various cortical areas as well as glial cells such as myelin-forming oligodendrocytes or astrocytes. Since the *in situ* hybridisation method did not permit cellular localisation of mRNA expression, the precise expression of either gene within this highly complex tissue could not be determined: whether the mRNA was expressed in glial

(oligodendrocytes or astrocytes) or neuronal cells (axonal RNA translation has been shown to occur both in mature and developmental axons; for review see Piper and Holt 2004). For this purpose, it may have been essential to achieve cellular resolution by coating the slides in photographic emulsion (Wisden and Morris 2002). An *a priori* similar expression pattern (such as that of *Edg2* and EST AI072720 under our conditions) may therefore reflect very different cellular expression and does not necessarily mean that both genes have similar or related functions. Moreover, in the context of schizophrenia, it may be that it is the expression of one gene within a particular region which is relevant to the psychopathology whereas it is another region for another gene. This study did not give enough information to provide any insight into the role of either gene.

Nevertheless, the expression pattern of *Edg2* was consistent with published literature showing prominent expression of *Edg2* mRNA and Edg2 protein within white matter tracts and specifically in oligodendrocytes both in human and rat adult brains (Allard *et al.* 1998; Allard *et al.* 1999; Handford *et al.* 2001; Cervera *et al.* 2002). Such an expression pattern in the adult brain as well as colocalisation with *myelin basic protein* (*Mbp*) strongly suggested that *Edg2* was involved in myelination and/or myelin maintenance during postnatal life (Allard *et al.* 1998; Handford *et al.* 2001; Cervera *et al.* 2002). However, even though its expression levels were much lower, *Edg2* was also expressed in cortical areas where it has been hypothesised to mediate the LPA-induced regulation of NMDA-receptor function (Lu *et al.* 1999; Yoshida and Ueda 2001). In particular, qRT-PCR analysis of *Edg2* expression in laser-microdissected prelimbic cortex provided confirmation that *Edg2* was expressed in this subpart of the prefrontal cortex which plays a major role in working memory (Antonio Ferra and Catherine Winchester).

Interestingly, both areas of expression and both functions may be related to schizophrenia. Therefore examining anatomical differences in *Edg2* expression after chronic PCP treatment turned out to be a useful strategy for elaborating a hypothesis about the potential role of *Edg2* in schizophrenia pathophysiology. However, failure to confirm the differential upregulation of *Edg2* expression in any cortical area and to show changes in expression in other regions of the brain did not provide any information of its role.

Nevertheless, at the same time as this analysis was performed, *Edg2* expression was also analysed by *in situ* hybridisation on tissue taken from animals that had been used previously (Egerton *et al.* 2005) to investigate the effects of chronic PCP administration on behaviour in the attentional set-shifting task (ASST), a task developed (Birrell and Brown 2000) for assessing the ability to shift perceptual attention forms in the rat as the equivalent to one of the core aspects of executive dysfunction in schizophrenia (Egerton *et al.* 2005). Again, there were no main effects of chronic PCP treatment on *Edg2* expression using this paradigm but interestingly, behavioural testing produced a significant increase in the expression levels of *Edg2* ($F_{(1,20)} = 4.98$, $p = 0.037$) in the infralimbic and prelimbic cortices and this effect was inhibited by chronic PCP treatment ($F_{(1,20)} = 6.08$, $p = 0.023$) in the infralimbic cortex (figure 3.12). No significant effects of drug treatment or behavioural testing were observed in all the white matter regions analysed. Because the attentional set-shifting task has been shown to be mediated by prefrontal cortical activity, the increased expression of *Edg2* in the prelimbic and infralimbic cortices suggested that neuronal *Edg2* expression was involved in cognitive-related activity. Although we did not confirm the differential upregulation of *Edg2* in the rat prefrontal cortex after chronic PCP treatment, this additional information provided evidence suggesting that neuronal *Edg2* may be more important for schizophrenia-related cognitive tasks than white matter *Edg2*.

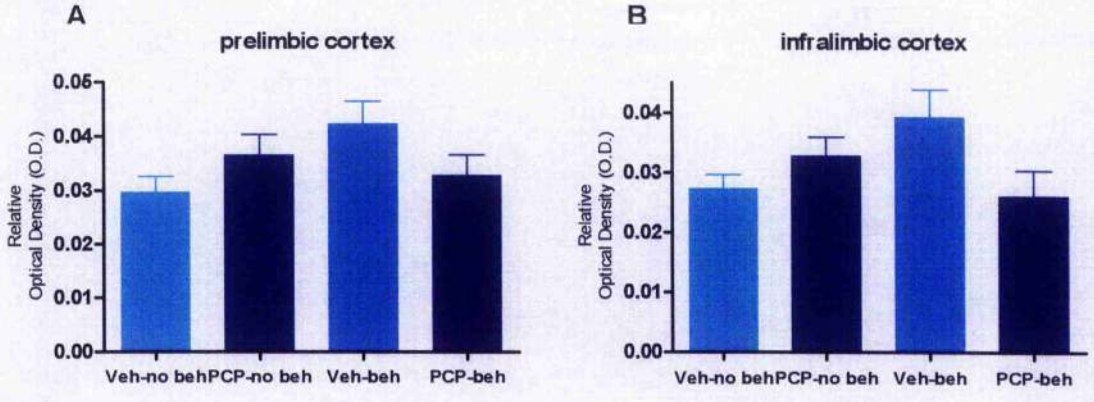


Figure 3.12. *Bar graphs for rat Edg2 in situ hybridisation (data from Alice Egerton)*

Results shown are mean ± standard error of the mean (s.e.m.)
n=6 animals for each of the 4 groups: vehicle-no behaviour, PCP-no behaviour, vehicle-behaviour and PCP-behaviour ("no behaviour" or "behaviour" meaning animals that were not behaviourally tested or had completed the attentional set-shifting task respectively).
A. prelimbic cortex, B. infralimbic cortex.

Further support of the potential involvement of neuronal *Edg2* in psychiatric disorders and particularly schizophrenia was provided by evidence showing that Edg2 knock-out mice displayed a region-specific disruption of markers of interneuronal function and interneuron-mediated brain rhythms. Thus, the loss of neuronal *Edg2* induced a similar network disruption to that observed acutely with NMDA receptor antagonism suggesting that neuronal *Edg2* may play an important role in schizophrenia pathophysiology (Cunningham *et al.* 2006).

In summary, although *in situ* hybridisation did not confirm the differential expression of both *Edg2* and the EST AI072720 after chronic PCP treatment, it allowed the determination of the expression pattern of both genes throughout the rat brain. Failure to validate the gene expression changes found in the microarray within cortical areas may be explained by the limited sensitivity of this technique. Indeed, although both X-ray film images and autoradiographic signals have been shown to be robust and equally applicable to semi-quantitative studies (Morris 1997; Simpson and Morris 2000), *in situ* hybridisation remains more a qualitative than a semi-quantitative method which may not be suitable or powerful enough for detecting low expression changes such as the ones detected in the chronic PCP model. Moreover, both *Edg2* and the EST AI072720 exhibited very low expression levels within cortical areas and especially the prefrontal cortex which made obtaining significant results even more difficult. Increasing the n number may have been necessary for improving the power of the study and increasing the chance of obtaining significant results.

In conclusion, the work presented in this chapter has allowed the identification and the validation of two candidates, *Edg2* and the EST AI072720, whose cortical expression and upregulation after chronic PCP treatment will be further characterised in relation with schizophrenia.

CHAPTER 4: CHARACTERISATION OF EDG2 AS A DRUG TARGET FOR SCHIZOPHRENIA

4.1- Introduction

Further pathway analysis of the microarray data was performed to elaborate a hypothesis about the potential role of Edg2 in schizophrenia. The initial analysis of the RG-U34A, B and C GeneChip® data reflecting differentially expressed genes in the rat prefrontal cortex after chronic PCP treatment was correlated with the analysis of a microarray investigating gene expression changes in the post-mortem dorso-lateral prefrontal cortex (DLPFC) of schizophrenic patients compared to controls (Hiromitsu Ozeki). This approach allowed the identification of a set of genes including *Ptk2b*, *Nck1* and *Mapk9* but also *Gab1*, *Gab2*, *Map4k4*, *Map3k7* and *MapK8ip1*, whose concomitant changes in expression were likely to reflect potential interactions within a pathway leading from Ptk2b (a non-receptor tyrosine kinase also referred to as proline-rich tyrosine kinase 2 or Pyk2) to Mapk9 activation (the "Pyk/Nck" pathway, pending British Patent application reference 0610574.6) and potentially compromised in PCP-treated rats and in human schizophrenic patients.
Moreover, activation of Ptk2b by LPA, probably *via* stimulation of Edg2 receptors, suggested that this G-protein coupled receptor for LPA, differentially expressed in the rat PFC after chronic PCP treatment and in post-mortem DLPFC of schizophrenic patients, may couple to the "Pyk/Nck" pathway leading to Mapk9 activation.
Figure 4.1 shows the potential functional relationship between all these genes. Further information on their expression changes in the rat and human microarrays can be found in Appendix C.

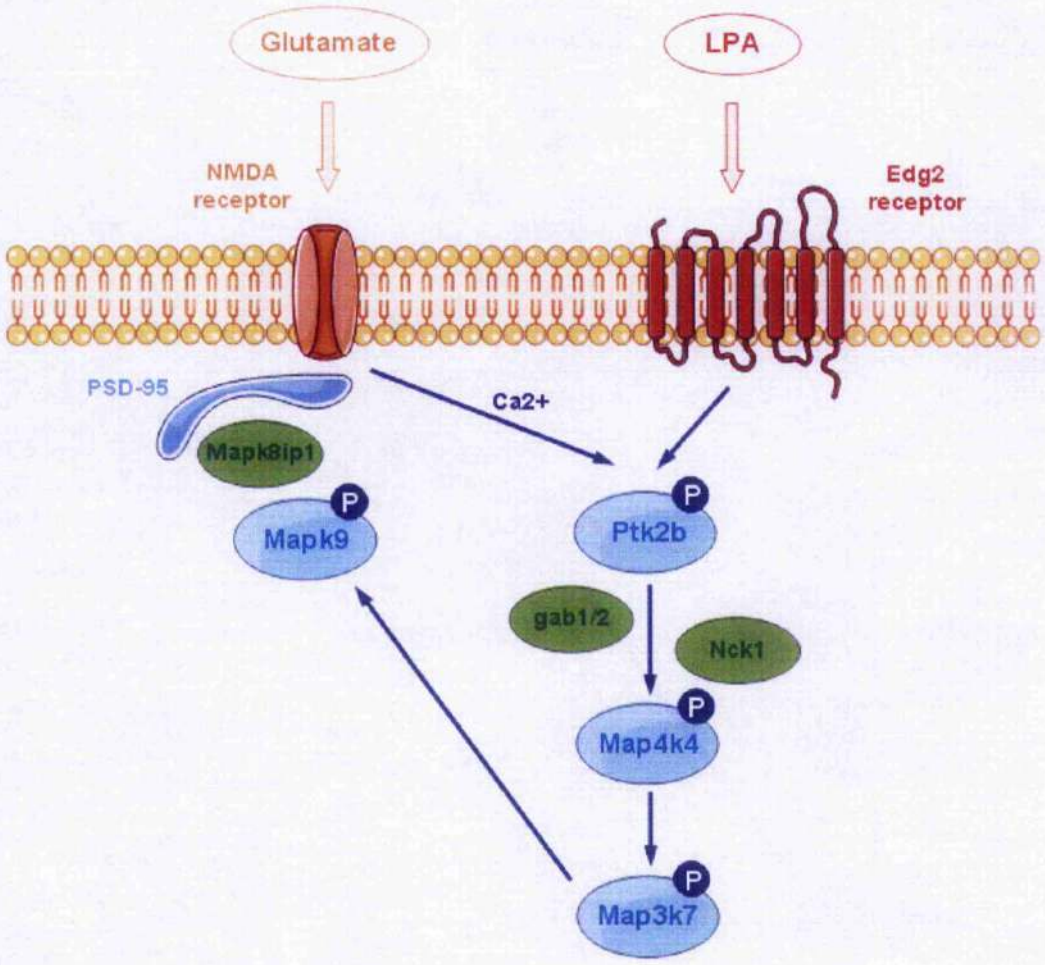


Figure 4.1. *Schematic representation of the functional relationship between Edg2 and "Pyk/Nck" pathway proteins*

The "Pyk/Nck" pathway downstream from NMDA receptors was suggested to be located downstream from Edg2 receptors so that stimulation of these receptors may increase downstream signal transduction. This pathway is structurally organised as a Mapk signalling cascade whereby the mitogen-activated protein kinase Mapk9 (Jnk2) is activated by the dual specificity mitogen-activated protein kinase kinases Map3k7/Map4k4 which are themselves activated by mitogen-activated protein kinase kinase kinases downstream from the protein tyrosine kinase Ptk2B. Gab1/2 and Nck1 are scaffold proteins that bring upstream and downstream kinases in close proximity ensuring an effective and specific signal transduction.

The hypothesis that the "Pyk/Nck" pathway may be implicated in schizophrenia pathophysiology (pending British Patent application reference 0610574.6) was elaborated based on literature showing that Ca^{2+} influx through post-synaptic NMDA receptors not only activates CamK but also stimulates neuronal gene expression *via* signalling pathways such as the MAPK signalling cascades (Haddad 2005).

Moreover, although coupling of NMDA receptors to the Erk family of Mapks is much more documented, activation of NMDA receptors has been shown to lead to the activation of the Jnk family of MAP kinases (Ko *et al.* 1998; Centeno *et al.* 2006; Centeno *et al.* 2007) so that NMDA receptor hypofunction may induce a decrease in Jnk (Mapk8, 9 and 10) activation as was observed in the rat chronic PCP model and in schizophrenic patients (figure 4.1). Consistent with this hypothesis, analysis of differentially expressed Mapk-related proteins revealed that most of them, including the MAP kinase kinase kinases (Mapkkks) Map3k9 (Mlk1) and Map3k7 (Tak1) and the upstream Map4k4 (Nck-interacting kinase) protein as well as interacting or scaffolding proteins such as Mapk8ip1 (Jip1), Nck1 and Gab1/2 were involved in the Jnk (Mapk8, 9 and 10) rather than the Erk signalling pathway (Johnson; Davis 2000; Johnson and Lapadat 2002; Vlahopoulos and Zoumpourlis 2004). This suggested that the Jnk pathway was compromised in the chronic PCP model and in schizophrenia, opposite to data showing a role for Erk but not Jnk proteins in NMDA receptor signalling and schizophrenia (Kyosseva *et al.* 2001). However major methodological differences (including PCP treatment regime) between these two studies and interactions between the Mapk signalling cascades (Shen *et al.* 2003; Waetzig and Herdegen 2005) suggested that these cascades were not mutually exclusive but were either together or individually differentially affected in schizophrenia, *e.g.* in different brain regions (Kyosseva *et al.* 2001).

Activation of the Jnk signalling cascade downstream of NMDA receptors has been shown to be mediated by Ptk2b (Pyk2) (also called cell-adhesion kinase β (CaKβ) or focal adhesion kinase 2 (Fak2)). Ptk2b, which is activated in response to a number of extracellular stimuli that increase Ca^{2+} such as NMDA receptor activation (Girault *et al.* 1999) was found to feed into and activate Mapk pathways (Lev *et al.* 1995; Dikic *et al.* 1996; Della Rocca *et al.* 1997) and more precisely to couple with the Jnk pathway to trigger Jnk phosphorylation (Tokiwa *et al.* 1996; Yu *et al.* 1996; Blaukat *et al.* 1999). Interestingly, Ptk2b was also suggested to be involved in the regulation of NMDA receptor activity as part of a cascade upstream of the NMDA receptors in which its activation by PKC may induce Src activation and potentiate NMDA receptor function by boosting the influx of Ca^{2+} through the receptor and setting in motion the downstream cascade (Girault *et al.* 1999; Huang *et al.* 2001; Salter and Kalia 2004; Alier and Morris 2005). Downregulation of *Ptk2b* expression in the rat

chronic PCP model and in schizophrenia was therefore consistent with the antagonism properties of PCP at the NMDA receptor and with the NMDA receptor hypofunction hypothesis of schizophrenia (Jentsch and Roth 1999; Olney *et al.* 1999).

Evidence suggesting that the LPA receptor Edg2 may couple to this Ptk2b-activated signalling cascade came from studies showing that Ptk2b can be activated by G protein-coupled receptor (GPCR) agonists including LPA (Dikic *et al.* 1996; Seufferlein *et al.* 1996; Andreev *et al.* 2001; Wu *et al.* 2002) and lead to Mapk activation (Kranenburg *et al.* 1999; Kranenburg and Moolenaar 2001). The effects of LPA mediated by Ptk2b include cell migration (Park *et al.* 2006) and neurite retraction and cell rounding (Kranenburg *et al.* 1999; Sayas *et al.* 2006), suggesting that Mapk activation may mediate the $G_{i/o}$ protein-mediated proliferative effects as well as the $G_{12/13}$ protein- and Rho-mediated morphological changes induced by LPA. Interestingly, coupling of LPA receptors to G_q proteins has been shown to initiate the cascade described previously whereby PKC-dependent activation of Ptk2b and Src can potentiate NMDA receptor currents (Tabuchi *et al.* 1997; Lu *et al.* 1999; Huang *et al.* 2001; Salter and Kalia 2004).
Coupling of Edg2 to different types of G proteins may therefore, via Ptk2b activation, both regulate NMDA receptor activity and induce Mapk activation. In addition, stimulation of all G_i, G_q and G_s-coupled receptors can lead to phosphorylation of Jnk, suggesting that G protein signals may be integrated at the level of Mapk, resulting in differential effects on Erk, Jnk and p38 Mapks (Chan *et al.* 2005).

Further analysis of the microarray results was therefore consistent with the literature, converging in the hypothesis that the Ptk2b signalling cascade downstream of both NMDA and Edg2 receptors was compromised in the rat chronic PCP model and in schizophrenic patients (figure 4.1). This pathway was termed the "Pyk/Nck" pathway since Nck1, an adaptor protein which was also found to be differentially expressed after chronic PCP treatment, had been shown to regulate the activation of Jnk through interaction with several proteins including the p21-GTPase activated kinase Pak1 (Mizuno *et al.* 2002; Poitras *et al.* 2003).

The upregulation of *EDG2* expression after chronic PCP treatment and in schizophrenic patients was suggested to represent a compensatory mechanism aiming at restoring NMDA receptor function by coupling to the same signalling cascade, the "Pyk/Nck" pathway. Interestingly, this hypothesis was consistent with the observed schizophreniform behaviour and with the schizophrenia-related neurochemical changes exhibited by *Edg2* knock-out mice (Harrison *et al.* 2003; Roberts *et al.* 2005).
Overall, Edg2 stimulation may therefore represent an additional route for regulating NMDA-mediated signal transduction events eventually leading to changes in neuronal gene transcription and synaptic plasticity (Thomas and Huganir 2004).

In order to test the hypothesis that modulating Edg2 activity may restore NMDA receptor function in schizophrenia, the *EDG2* human cDNA was cloned into an expression vector and an *EDG2*-overexpressing stable cell line was generated. The ability of EDG2 overexpression to restore activity of the "Pyk/Nck" pathway was investigated *in vitro* by looking at gene expression changes within the cascade as well as measuring phosphorylation of Mapk9 (its final output), Mapk8 and Mapk10. As EDG2 was emerging as a potentially attractive drug target, a [^{35}S]-GTPγS binding assay was developed using the *EDG2*-overexpressing stable cell line to allow screening of potential EDG2 agonists *in vitro*.

4.2- *EDG2* construct generation and development of a stable transformed cell line

To characterise functionally the role of *EDG2* in relation to the "Pyk/Nck" pathway and schizophrenia, *in vitro* studies were undertaken and as a first step *EDG2* was cloned into pMSF1, an expression vector based on pcDNA3.1-FLAG® whose N-terminal signal sequence helps the anchoring of the cloned gene into the membrane (Mitsubishi Pharma Corporation).

A human full-length *EDG2* cDNA clone was purchased as a plasmid (pBluescriptR) in *E. coli* and its identity was verified by DNA sequencing as being identical to

Genbank mRNA sequence NM_001401 (or NM_057159), *i.e.* the differentially expressed isoform of *EDG2* identified by the microarray analysis of human DLPFC of schizophrenic patients (Hiromitsu Ozeki). Primers containing 5'- and 3'-end restriction enzyme recognition sites (for *EcoRV* and *XhoI* respectively) were designed to allow in frame cloning of *EDG2* cDNA into pMSF1 expression vector and were used to amplify *EDG2* from its original pBluescriptR vector.

The PCR-amplified product of the expected length and the pMSF1 vector were digested by *EcoRV* and *XhoI* and the approximately 1.1kb *EDG2* cDNA was cloned into the expression vector.

TOP10 bacterial cells were transformed with pMSF1-*EDG2* construct (section 2.5.1) and the cells were checked for the presence of the expression construct. Plasmid DNA was extracted from twenty-four colonies and digested with *EcoRV* and *XbaI* to screen the plasmids for the *EDG2* insert. One of the plasmids was shown to contain the *EDG2* insert. Sequencing of plasmid DNA across the vector/insert junction confirmed the correct in frame insertion of the *EDG2* cDNA within the pMSF1 vector while sequencing of the entire *EDG2* cDNA insert confirmed its identity as identical to Genbank mRNA sequence NM_001401.

In order to overexpress *EDG2 in vitro*, human SK-N-SH cells were transfected with the pMSF1-*EDG2* expression construct and empty pMSF1 vector (negative control for subsequent functional studies) (section 2.5.4.1). Cell lysate was extracted after 48h (section 2.5.6) and proteins were quantified using the Bradford dye-binding assay (section 2.5.8).

FLAG® western blotting on cell lysate from pMSF1-*EDG2* transfected cells was used first to check the overexpression of the FLAG®-EDG2 fusion protein. Both the methodology and the FLAG® antibody were shown to be effective by using a positive control consisting of another FLAG® fusion protein of known size but they failed to confirm the overexpression of the FLAG®-EDG2 fusion protein from pMSF1-*EDG2* (figure 4.2). However, subsequent thorough examination of the pMSF1 sequence revealed the presence of only 7 out of the 8 amino acids (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) representing the FLAG® epitope which may be insufficient to allow specific binding of the FLAG® antibody to this epitope and may therefore prevent the detection of the fusion protein. This possibility may also explain the non-detected overexpression of the empty pMSF1 expression vector,

although it is more likely that the FLAG® epitope had not been translated because it was not in fusion with another protein.

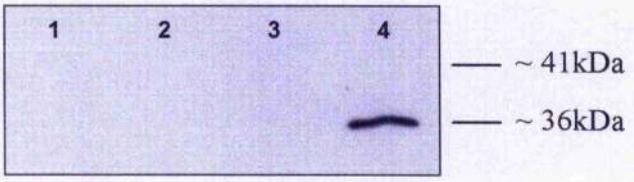


Figure 4.2. *FLAG® western blotting on pMSF1-EDG2-transfected SK-N-SH cell lysate*

Lanes 1 and 2 represent SK-N-SH cells transfected with pMSF1-*EDG2* construct (translating into a ~41kDa protein) and the empty pMSF1 vector respectively. Lane 3 is a no protein negative control and lane 4 is a positive control consisting of a cell lysate from pcDNA3.1-FLAG®-*NCK1* transfected SK-N-SH cells (MW~36kDa). A FLAG® M2 antibody was used at a 1/5000 dilution (section 2.5.9).

As a consequence, western blotting using EDG2 antibodies was performed on pMSF1-*EDG2* transfected SK-N-SH cells to confirm the overexpression of EDG2 *in vitro*. However none of the EDG2 antibodies utilised detected EDG2 (endogenous or overexpressed FLAG®-EDG2) in the pMSF1-*EDG2* or pMSF1 transfected cells. Many conditions were tried to optimise use of the EDG2 antibodies (transfection, cell lysis, antibody as well as western blotting visualisation substrate).

Experimental condition	Experimental details
Transfection reagents and conditions	Lipofectamine 2000 (Invitrogen) was tried in addition to Mirrus *TransIT*-LT1. For each of these transfection reagents, different amounts of DNA and different ratios DNA/reagent were tried.
Cell lysis buffers	Four different buffers were tried, including two different RIPA buffers (among them one following Upstate's recipe), a buffer prepared following Cell Signalling's recipe and one following a recipe used in a paper on EDG2 (Fukushima *et al.* 1998).
EDG2 antibodies	5 EDG2 antibodies were tried, from Abcam, Upstate, Abgent, Exalpha Biologicals and Sigma.
FLAG® antibody	A FLAG® antibody was tried on cell lysates obtained with different buffers and following various transfection conditions.
Visualisation substrates	Pierce SuperSignal West Femto Maximum Sensitivity Substrate was used in addition to ECL Plus detection System (Amersham).

Table 4.1. *Summary of different conditions tried to optimise western blotting of EDG2 protein*

The transfection efficiency issue was ruled out by performing qRT-PCR on cDNA synthesised from mRNA extracted from pMSF1-*EDG2*-transfected SK-N-SH cells. Three different ratios of DNA/transfection reagent were tried (from 1µg DNA for 2µl reagent up to 1µg DNA for 6µl reagent. Figure 4.3 shows the amplification plot of this qRT-PCR.

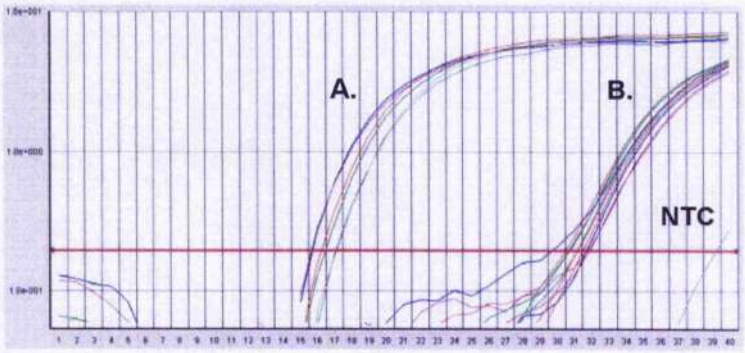


Figure 4.3. *Amplification plot from ABI SDS 7000 Real-Time PCR machine*

X-axis is the PCR cycle number, Y-axis is the neperian logarithm (ln) of the fluorescence. Curves shifted to the left, showing more fluorescent product at lower cycle number reflect more PCR product. Each curve represents one sample amplified in duplicate. The 'A' group of curves represents SK-N-SH cells transfected with pMSF1-*EDG2* construct using different ratios of DNA to transfection reagent and the 'B' group represents controls, including SK-N-SH cells transfected with pMSF1 and untransfected SK-N-SH cells. Therefore qRT-PCR amplification of *EDG2* at later cycle numbers in pMSF1-transfected and untransfected SK-N-SH cells ('B' group of cells) reflects endogenous *EDG2* expression in these cells while *EDG2* overexpression was confirmed by earlier qRT-PCR amplification of *EDG2* in pMSF1-*EDG2* transfected cells ('A' group of cells). NTC (non-template control) represents the amplification plot of the qRT-PCR reaction performed using H_2O but no cDNA, so that no or very late amplification indicates absence of contamination

pMSF1-*EDG2* transfected SK-N-SH cells were found to show a greater amount of *EDG2* transcript, represented by earlier detection of the *EDG2* amplicon (Figure 4.3, 'A' curves), than non-transfected SK-N-SH cells (Figure 4.3, 'B' curves). Moreover, the three different ratios used for the transfection induced slight variations in amplification of *EDG2*, which allowed us to select the best ratio to use for subsequent studies. 1µg DNA to 6µl reagent was thus determined to be the ratio that induced the higher *EDG2* expression (cells transfected using this ratio were found to express *EDG2* approximately 1.75×10^5 times more than wild-type SK-N-SH cells).

In addition, the qRT-PCR showed that wild-type SK-N-SH cells express endogenous *EDG2*.

This experiment therefore confirmed the overexpression of *EDG2* mRNA after transfection of SK-N-SH cells with pMSF1-*EDG2*. It was therefore assumed that the pMSF1-*EDG2* transfected cells did indeed overexpress the EDG2 protein.

However, although preliminary functional studies were undertaken using these transiently *EDG2*-overexpressing cells, the inherent variability in their levels of expression of *EDG2* due to the unavoidable unevenness in the transfection efficiency encouraged the development of an *EDG2*-overexpressing stable cell line. Creation of such a cell line was also motivated by promising preliminary functional results obtained using transient *EDG2*-overexpressing cells (section 4.3) and by promising *in vivo* data obtained concomitantly in the lab by Alice Egerton, Susan Cochran and Lee Reid (section 4.7.2). These latter results, providing further evidence that EDG2 may potentially represent a promising drug target for schizophrenia, reinforced the need for an *EDG2*-overexpressing cell line of constant phenotype, essential for further functional studies and for screening purposes

An *EDG2*-overexpressing stable cell line was thus developed by treating transiently *EDG2*-overexpressing SK-N-SH cells with 1mg/ml gentamycin (G418) and isolating surviving *EDG2*-overexpressing clones. After several rounds of selection, 15 clones were obtained and the levels of *EDG2* mRNA expression were checked using qRT-PCR. On the PCR amplification plot (data not shown), 7 clones were found to exhibit higher *EDG2* expression levels than wild-type SK-N-SH cells while 8 clones did not show any increase in *EDG2* expression. *EDG2* expression levels were normalised to those of an internal control, *GAPDH*, in order to remove any potential variability due to different amounts of starting cDNA, and 3 clones were eventually found to show higher *EDG2* expression levels than wild-type cells (Figure 4.4).

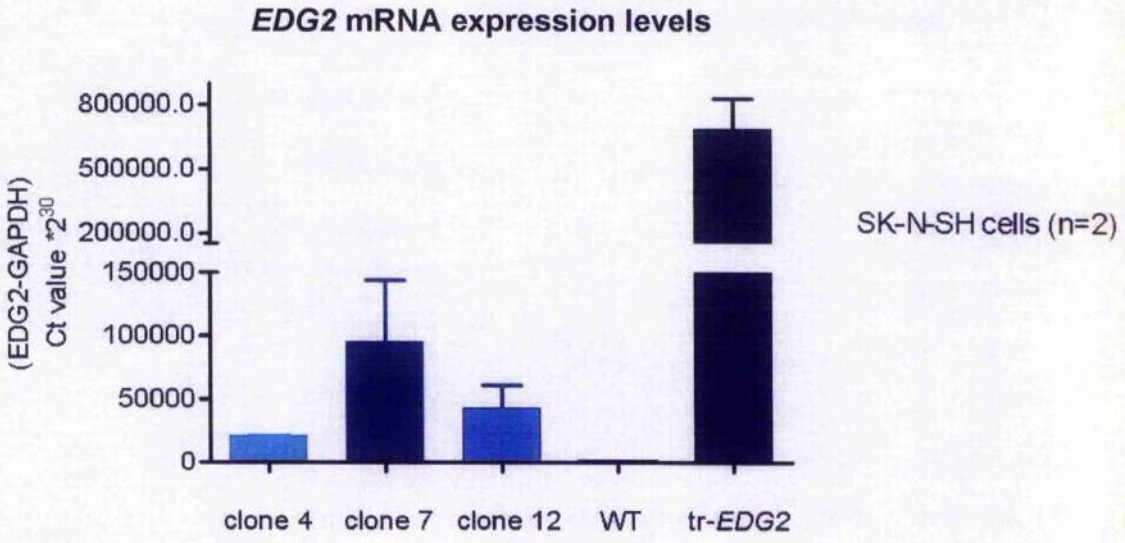


Figure 4.4. *Bar graph bar showing EDG2 expression levels*

Bars represent *EDG2* expression levels in stable clones 4, 7 and 12 and wild-type SK-N-SH cells (WT) and SK-N-SH cells transiently overexpressing *EDG2* (tr-*EDG2*). n=2 for each condition. Data from qRT-PCR are expressed as mean ± standard error of the mean (s.e.m).

Of these 3 clones, clone 7 displayed the highest *EDG2* expression, which was about 7 times less than SK-N-SH cells transiently overexpressing *EDG2* but almost 25,000 times more than wild-type SK-N-SH cells. This clone was therefore confirmed to overexpress *EDG2* receptors.

4.3- Preliminary functional studies using transiently *EDG2*-overexpressing cells

4.3.1- JNK-P western blotting

Preliminary studies were performed using SK-N-SH cells transiently overexpressing *EDG2* to investigate the potential activation of the "Pyk/Nck" signalling cascade following LPA treatment. Phosphorylation (hence activation) of the final output of this pathway, MAPK9 (JNK2), was assessed by western blotting using a phospho-JNK antibody. This antibody recognises more than one JNK isoforms. On western blots it detects two bands of ~46 and 54 kDa corresponding approximately to

MAPK8 (JNK1) and MAPK9 (JNK2) respectively, which allowed the analysis of the cellular response induced by LPA treatment in terms of pathway activation.

Cell lysate from SK-N-SH cells transfected with pMSF1-*EDG2* and treated prior to lysis with 1μM LPA were thus analysed for JNK phosphorylation. 40μg protein was subjected to electrophoresis in a 10% (w/v) SDS-PA gel and transferred by western blotting to PVDF membrane. Immunodetection was performed with the JNK-P antibody and after visualisation, membranes were wet with methanol, stripped and reblotted with an actin antibody to confirm even loading of proteins (figure 4.5,B).
Visual examination of the JNK-P blots (Figure 4.5) suggested that *EDG2* overexpression (lanes 4 and 9 respectively compared to lanes 3 and 8) as well as LPA stimulation (lanes 6 to 9 compared to lanes 1 to 4) induced an increase in the phosphorylation levels of both JNK_{46kDa} and JNK_{54kDa}.

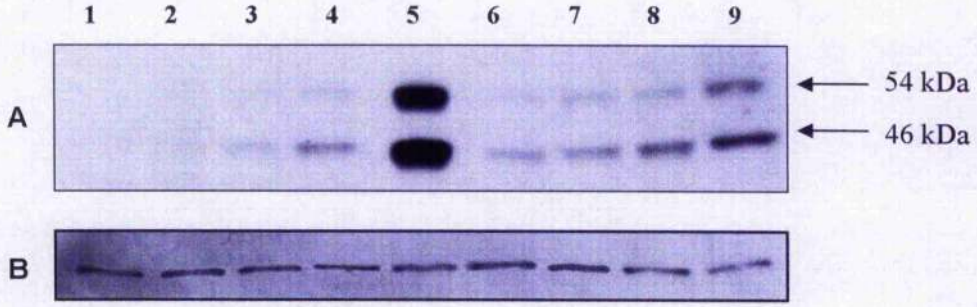


Figure 4.5. *Western blot showing JNK-P activation after EDG2 overexpression in SK-N-SH cells. A. JNK blot; B. actin blot.*

Lane 5 is a positive control consisting of UV-treated SK-N-SH cells (UV is known to strongly activate JNK). Lanes 1 to 4 show JNK-P expression in cells treated under the same conditions as in lanes 6 to 9 except that cell lysates run in lanes 6 to 9 were from cells treated with 1μM LPA for 30 minutes whereas cells run in lanes 1 to 4 were treated with vehicle (PBS + 0.1% BSA). Lanes 1 and 6 represent wild-type SK-N-SH cells treated with vehicle, lanes 2 and 7 represent SK-N-SH cells treated with the transfection reagent only, lanes 3 and 8 represent SK-N-SH cells transfected with the empty pMSF1 vector and lanes 4 and 9 represent pMSF1-*EDG2* transfected SK-N-SH cells.
These autoradiographs are representative of these experiments that were repeated 4 times (section 2.5.9). The strongest bands represent JNK-P activation (by UV stimulation in lane 5 and in pMSF1-*EDG2* transfected cells in lane 9).

JNK-P_{46kDa} and JNK-P_{54kDa} as well as actin expression levels were quantified with densitometry. JNK-P levels normalised to actin were analysed by ANOVA (Figure 4.6)

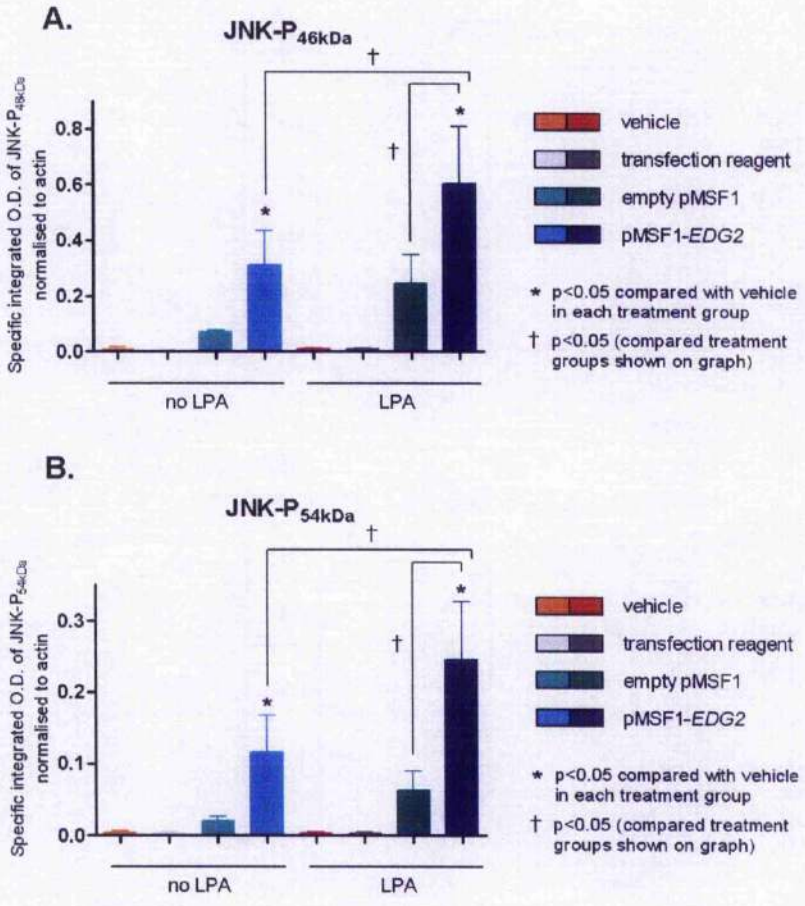


Figure 4.6. *Bar graphs showing JNK-P_{46kDa} (A) and JNK-P_{54kDa} (B) levels after LPA treatment*

Samples (n=4 replicates) represent SK-N-SH cells treated with a vehicle, SK-N-SH cells treated with the transfection reagent, pMSF1-transfected cells and pMSF1-*EDG2*-transfected cells. Data are expressed as mean ± standard error of the mean (s.e.m). Data were analysed by ANOVA using LPA treatment (1μM LPA for 30 minutes) and transfection status (cells treated with PBS as vehicle, pMSF1-transfected cells or pMSF1-*EDG2*-translated cells) as the factors (n=4). Transfection status was found to have a significant effect on both JNK-P_{46kDa} ($F_{(1,3)}$=10.55, $p<0.001$) and JNK-P_{54kDa} ($F_{(1,3)}$=11.362, $p<0.001$). Significant interactions were further explored by simple main effects analysis (multiple comparisons were adjusted using Bonferroni's procedure). * signifies $p<0.05$ compared with vehicle in each treatment group; † signifies $p<0.05$ for comparisons between group treatments as shown on graph (Bonferroni-adjusted pairwise comparisons).

Analyses of JNK-P levels confirmed the visual observations of the blots (example shown in Figure 4.5), suggesting that both JNK_{46kDa} (figure 4.6.A) and JNK_{54kDa} (figure 4.6.B) were activated by *EDG2* overexpression. Overall, activation of JNK proteins of both molecular weights by LPA stimulation did show a similar pattern and statistical analyses also proved to yield consistent results. Thus, for both groups of JNK proteins, ANOVA analyses revealed a very large effect of the transfection status (including *EDG2* overexpression) on JNK phosphorylation overall (p values < 0.001 for JNK-P_{46kDa} and JNK-P_{54kDa}). Conversely, LPA treatment did not have a significant effect on JNK activation overall ($p=0.086$ for JNK-P_{46kDa} and $p=0.105$ for JNK-P_{54kDa}) but in *EDG2*-overexpressing cells only (as revealed by post-hoc pairwise analyses; $p=0.036$ for JNK-P_{46kDa} and $p=0.016$ for JNK-P_{54kDa}). No interaction was found between transfection status and LPA treatment for either or both groups of JNK proteins.

Consistent with these overall analyses, post-hoc pairwise analyses revealed that *EDG2* overexpression induced a significant increase in the phosphorylation of JNK_{46kDa} and JNK_{54kDa} when compared individually to non-transfected cells treated with a vehicle or with the transfection reagent or with cells transfected with the empty pMSF1 expression vector. The only comparison which did not reach significance was the one examining JNK phosphorylation in pMSF1-*EDG2* transfected cells compared to pMSF1-transfected cells in absence of LPA treatment ($p=0.076$ for JNK-P_{46kDa} and $p=0.067$ for JNK-P_{54kDa}). Conversely, p values in the 0.001 range or < 0.001 were found for all individual comparisons of JNK-P_{46kDa} and JNK-P_{54kDa} levels between LPA-treated *EDG2*-overexpressing cells and LPA-treated non-transfected cells suggesting that LPA-stimulation of overexpressed *EDG2* receptors resulted in a major increase in JNK_{46kDa} and JNK_{54kDa} phosphorylation.
The observed slight increase in JNK phosphorylation in pMSF1-transfected cells compared to the two groups of non-transfected cells was not statistically significant and was not affected by LPA treatment which was consistent with the hypothesis that JNK activation in these conditions was due to DNA transfection-related stress rather than coupling of LPA to any receptor. These results further confirmed that significant increased phosphorylation of JNK proteins in *EDG2*-overexpressing cells was mediated by EDG2 receptors stimulated with LPA.

4.3.2- JNK-P ELISA

In order to confirm the results obtained by analysing JNK activation by western blot on *EDG2*-overexpressing cells, a JNK-P ELISA (Enzyme Linked Immunosorbent Assay) assay was used as a semi-quantitative method of measuring JNK phosphorylation. Similar to the antibody used for the western blots, this assay did not have the power to discriminate between JNK isoforms and equally detected MAPK8 (JNK1), MAPK9 (JNK2) and MAPK10 (JNK3). Despite this fact, the ELISA analysis of JNK/MAPK activity should be more reliable and accurate than western blot analysis.

Cell lysates from SK-N-SH cells transfected with pMSF1-*EDG2* and treated prior to lysis with vehicle (PBS + 0.1% BSA) or 1μM LPA were thus analysed for JNK phosphorylation using Pathscan Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA Kit (Cell Signalling). The assay was performed following the manufacturer's protocol using 15μg total protein. Figure 4.7 shows bar graphs representing absorbance at 450nm as a measure of the amount of phosphorylated SAPK/JNK proteins.

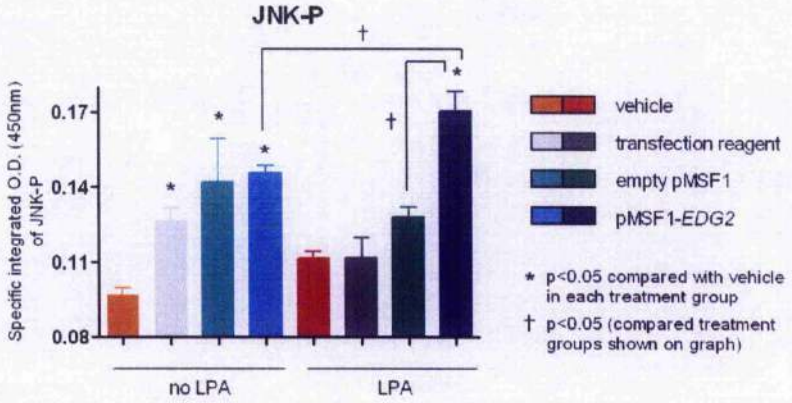


Figure 4.7. *Bar graph showing JNK-P levels after LPA treatment*

Samples (n=3) represent SK-N-SH cells treated with a vehicle, SK-N-SH cells treated with the transfection reagent, pMSF1-transfected cells and pMSF1-*EDG2*-transfected cells. Data are expressed as mean ± standard error of the mean (s.e.m). Data were analysed by ANOVA using LPA treatment (1μM for 30 minutes) and transfection status as factors, revealing a significant effect of transfection status ($F_{(1,2)}$=15.799, $p<0.001$). Further potential interactions were investigated by simple main effect analysis (multiple comparisons were adjusted using Bonferroni's procedure). * signifies $p<0.05$ compared with vehicle in each treatment group; † signifies $p<0.05$ for comparisons between group treatments as shown on graph (Bonferroni-adjusted pairwise comparisons).

Visual observation of JNK-P levels measured using the JNK-P ELISA assay appeared consistent with previous western blot analyses performed under the same conditions, with LPA inducing a general increase in JNK-P phosphorylation and *EDG2* overexpression further increasing its activation particularly in LPA-treated cells. The most apparent difference lay in the substantial (*versus* hardly detectable by western blot) endogenous levels of JNK phosphorylation detected by the JNK-P ELISA assay in non-transfected cells.

ANOVA analysis of JNK-P levels revealed a significant effect of transfection status (including *EDG2* overexpression) on JNK phosphorylation ($p<0.001$) but no effect of LPA treatment ($p=0.568$), which was consistent with western blot analyses. There was a significant interaction between transfection status and LPA treatment ($p=0.023$) suggesting that both factors were not completely independent.
Consistently with western blot analysis, simple main effects analyses revealed that LPA treatment induced a significant increase in JNK phosphorylation in *EDG2*-overexpressing cells ($p=0.012$) but not in any other group of non-transfected cells.
A significant effect of treatment with the transfection reagent and transfection with pMSF1 compared with treatment with a vehicle was found in absence of LPA treatment while *EDG2* overexpression did not induce further significant increase in JNK-P activation in these cells. Activation of JNK by transfection-related and especially by DNA transfection (pMSF1 transfection) may not be surprising since JNK is known to be preferentially and strongly activated by environmental stresses. The limited availability of LPA in the medium may explain that *EDG2* overexpression itself did not induce a significant activation of JNK while this activation was completely revealed in LPA-treated cells by the stimulation of overexpressed *EDG2* receptors with their endogenous ligand ($p<0.001$ comparing *EDG2*-overexpressing cells and pMSF1-overexpressing cells).

In conclusion, these results showed that *EDG2* overexpression and stimulation of EDG2 receptors by LPA induced a significant increase in JNK-P activation, which confirmed the ANOVA analyses of western blot experiments performed under the same conditions (section 4.3.1). Although these results were not restricted to any specific MAPK, they provided evidence that LPA stimulation of EDG2 receptors resulted in the final activation of MAPK proteins including MAPK9 which was

consistent with the hypothesis that EDG2 may provide an amenable route to modulate the activity of the "Pyk/Nck" pathway.
Striking similarity but improved sensitivity of these results also provided evidence that JNK-P ELISA could be favourably used instead of western blots to measure JNK-P activation.

4.4- Investigation of JNK-P activation in a stable transformed *EDG2*-overexpressing cell line

Preliminary evidence showing activation of MAPK9 (among other MAP kinases) after transient transfection of EDG2 receptors was consistent with the hypothesis suggesting that EDG2 stimulation may provide a way to restore activity of the "Pyk/Nck" pathway.
In this context, stable *EDG2*-overexpressing cells may be used as a tool for screening EDG2 ligand compounds. In order to validate the use of these cells in functional studies, the phospho-SAPK/JNK ELISA assay (section 4.3.2) was used to semi-quantitatively measure MAPK8/9/10 phosphorylation as an indication of the final output of the "Pyk/Nck" pathway in these cells.

Cell lysate from *EDG2*-overexpressing clones 4, 7 and 12 (section 4.2) and wild-type SK-N-SH cells were treated prior to lysis with 1μM LPA or vehicle and analysed for JNK phosphorylation. As for transient *EDG2*-overexpressing cells, 15μg total protein was used and the Pathscan Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA assay (Cell Signalling) was performed following the manufacturer's protocol. Figure 4.8 shows bar graphs representing absorbance at 450nm.

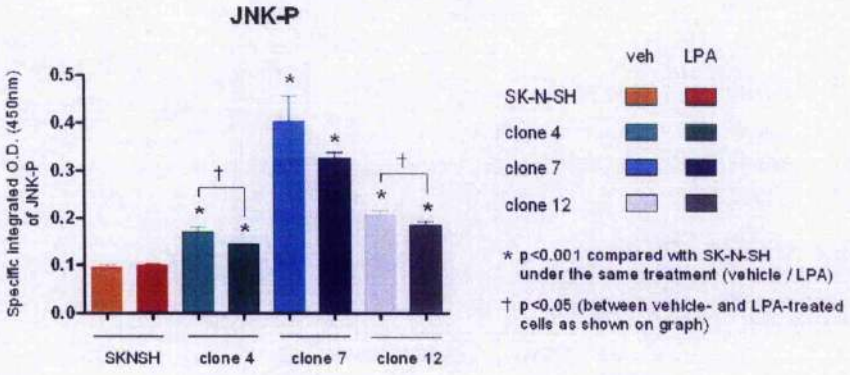


Figure 4.8. *Bar graph showing JNK-P levels in stable EDG2-overexpressing clones after LPA treatment*

Samples (n=3) represent wild-type SK-N-SH cells and *EDG2*-overexpressing clones 4, 7 and 12 treated with a vehicle or with LPA. Data are expressed as mean ± standard error of the mean (s.e.m). Data were analysed by ANOVA using LPA treatment and cell type as factors, revealing a significant effect of cell type ($F_{(1,3)}$=156.37, $p<0.001$). Further potential interactions were investigated by simple main effect analysis (multiple comparisons were adjusted using Bonferroni's procedure). * signifies $p<0.001$ compared untransfected SK-N-SH under the same treatment (vehicle or LPA); † signifies $p<0.05$ between vehicle- and LPA-treated cells as shown on graph (Bonferroni-adjusted pairwise comparisons).

Visual examination of the graph revealed an increased phosphorylation of JNK proteins in each of the 3 clones compared with wild-type SK-N-SH cells, clone 7 displaying highest JNK phosphorylation. Interestingly, levels of JNK-P activation in the 3 clones (clone 7 > clone 12 > clone 4) were consistent with *EDG2* expression levels in these clones as measured by qRT-PCR (figure 4.4).

ANOVA analysis of JNK-P levels revealed similar results for *EDG2*-overexpressing clones 4, 7 and 12. In each of these clones considered individually, *EDG2* overexpression significantly increased JNK phosphorylation independently of LPA treatment. Conversely, LPA treatment did not affect overall JNK activation in any of the clones, but it induced a small but significant decrease in JNK phosphorylation in *EDG2*-overexpressing clones 4 and 12. This result was inconsistent with previous data showing an increase in JNK phosphorylation in LPA-treated *versus* vehicle-treated cells transiently overexpressing *EDG2* (section 4.3.2). However, the LPA-induced decreased JNK phosphorylation was not significant for clone 7, which displayed highest *EDG2* expression levels (section 4.2). Moreover, JNK

phosphorylation levels shown by this clone after LPA treatment were still massively higher than those of wild-type SK-N-SH cells ($p<0.001$).

In conclusion, these results confirmed that stable *EDG2* overexpression induced the activation of (a) signalling pathway(s) leading to JNK phosphorylation. Among three stable *EDG2*-overexpressing clones, clone 7 displayed the highest JNK activation corresponding with the highest *EDG2* expression (section 4.2) therefore it was considered as the most promising for functional investigation of the "Pyk/Nck" pathway and for potential screening purposes.

4.5- qRT-PCR analysis of other components of the "Pyk/Nck" pathway

qRT-PCR was chosen as a method to perform a preliminary investigation of the potential activation of the "Pyk/Nck" pathway upstream of MAPK after *EDG2* overexpression. Potential gene expression changes of several components of this cascade including *NCK1*, *PAK1* (NCK1-interacting protein), *PTK2B* and *MAPK9* itself (Figure 4.1), were thus evaluated using stable *EDG2*-overexpressing clones. Although the methodology (qRT-PCR) and the design (absence of LPA treatment) of this experiment strongly limited its power and the probability to show any significant results, it was intended that these data may provide an insight into the activation of the "Pyk/Nck" pathway and the interactions between its different components.

In order to minimise the potential effect of other factors and ensure consistency between the groups, expression levels of "Pyk/Nck" pathway genes in *EDG2*-overexpressing clones 4, 7 and 12 were compared with their expression levels in three clones (clones 3, 10 and 13) which, although resistant to G418, displayed *EDG2* expression levels comparable to those of wild-type SK-N-SH cells. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), which exhibited unchanged expression in *EDG2*-overexpressing clones, was used as a covariate in the ANCOVA analysis of potential changes in *NCK1*, *PAK1*, *PTK2B* and *MAPK9* expression induced by *EDG2* overexpression.

Expression levels of *PAK1*, *PTK2B* and *MAPK9* were unchanged in *EDG2*-overexpressing clones compared to controls (data not shown) which may not be surprising since increased activity of these proteins (which are all kinases) occurs through and thereby may be reflected by increased phosphorylation and not by changes in mRNA expression. Thus, unchanged expression of *MAPK9* was expected since this kinase had been shown to be phosphorylated –hence activated- in *EDG2*-overexpressing clones (section 4.4) under the same conditions.

Conversely, there was some evidence that *EDG2* overexpression induced a decrease in *NCK1* expression (figure 4.9) which would confirm a link between EDG2 receptors and the "Pyk/Nck" signalling cascade.

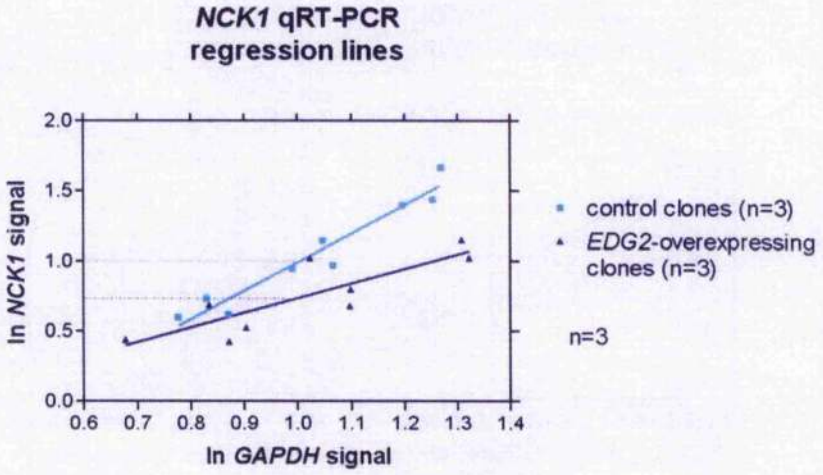


Figure 4.9. *Regression lines for NCK1 qRT-PCR of EDG2-overexpressing clones*

qRT-PCR was performed to evaluate *NCK1* expression changes in *EDG2*-overexpressing clones 4, 7 and 12 by comparison with clones 3, 10 and 13 (exhibiting expression levels of *EDG2* equivalent to those of wild-type SK-N-SH cells) (section 2.4.5). Y-axis is the logarithm of the fluorescence intensity representing the expression of *NCK1* and X-axis is the logarithm of the fluorescence intensity for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the internal control gene that was used for normalisation. For each of these genes, this graph plots three points taken from the linear amplification phase of the PCR. The data were not analysed by ANCOVA since the analysis assumes a constant relationship between target gene and covariate in both treatment groups (*i.e.* the lines should be parallel).

In conclusion, this study provided further evidence that *EDG2* overexpression affected the activity of the "Pyk/Nck" pathway consistently with the hypothesis that modulating EDG2 activity may constitute a way to alter the activity of this cascade. However, since like most signalling cascades, the "Pyk/Nck" pathway is a series of kinases, better characterisation of its activity and regulation may be achieved by looking at phosphorylation of its different components in *EDG2*-overexpressing clones and in response to LPA.

4.6- [^{35}S]-GTPγS binding assay on stable transformed *EDG2*-overexpressing cells

In order to allow screening of EDG2 ligand compounds *in vitro*, a [^{35}S]-GTPγS binding assay monitoring guanine nucleotide exchange at G-proteins upon agonist stimulation was developed using stable *EDG2*-overexpressing cells. Since the specificity of this assay was inherently limited by the specificity of LPA towards EDG2 receptors as compared to other GPCRs, it was essential to investigate the endogenous expression of other LPA receptors, namely EDG4, EDG7 and GRP23, in the cells that were being used in the assay. qRT-PCR was used as a sensitive method of assessing mRNA expression of these receptors in SK-N-SH cells, thereby enabling the specificity of [^{35}S]-GTPγS binding assay towards the exogenous and endogenous EDG2 receptors to be predicted. Using this technique, *EDG2*-overexpressing SK-N-SH cells did not show any expression of *EDG4* and *EDG7* and displayed very low endogenous *GPR23* expression (data not shown) suggesting that a LPA-induced [^{35}S]-GTPγS binding assay using these cells would be quite specific for EDG2 receptors.

The development of a [^{35}S]-GTPγS binding assay using *EDG2*-overexpressing cells required optimisation of the cellular membrane preparation and of the [^{35}S]-GTPγS binding assay and many attempts were necessary to obtain an assay giving consistent and reproducible results. Finally, the [^{35}S]-GTPγS binding assay was performed using a protocol adapted from Im *et al.* (2000) and a series of experiments were performed to obtain an LPA dose-response curve for each "type" of cells, *i.e.* wild

type SK-N-SH cells and *EDG2*-overexpressing clones 4, 7 and 12. As in most papers on LPA receptors including EDG2, final concentrations of LPA ranged from 0.1nM to 10μM LPA. Figure 4.9 shows LPA dose-response curves for SK-N-SH cells and *EDG2*-overexpressing clones 4, 7 and 12. Analysis of these curves by non-linear regression analysis with a sigmoidal dose-response curve fitting allowed the pEC_{50} of LPA (log_{10} concentration of LPA required to give 50% of its own maximal stimulation) to be calculated in each type of cell as a measure of potency (Table 4.2).

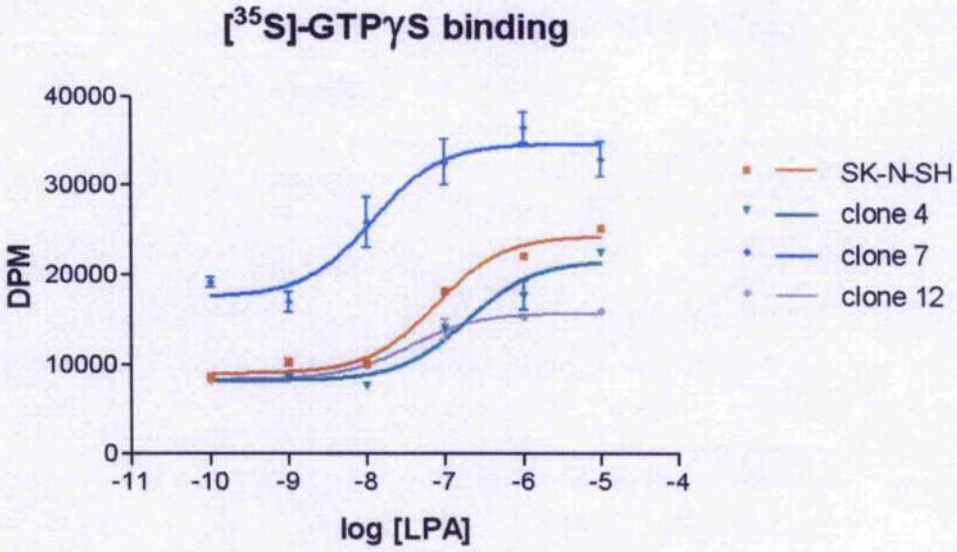


Figure 4.10. LPA-induced [^{35}S]-GTPγS binding

Dose-response curves represent LPA-induced [^{35}S]-GTPγS binding mediated by endogenous EDG2 receptors in SK-N-SH cells (■) and by endogenous and exogenous EDG2 receptors in clones 4 (▼), 7 (◆) and 12 (●) (n=2 for each cell type). [^{35}S]-GTPγS binding was performed following a protocol adapted from Im *et al.* (2000) using 25μg of total protein per reaction tube and final concentrations of 0.1nM, 1nM, 10nM, 100nM, 1μM and 10μM LPA. As assessed by comparison with the total amount of [^{35}S]-GTPγS introduced per reaction (data not shown), the radioactivity counts (in dpm: disintegrations per minute) obtained after filtration revealed that more than 10% of radioactivity was retained, making these results very reliable. Background [^{35}S]-GTPγS binding was subtracted from the data which were normalised to the maximal binding displayed by SK-N-SH cells when stimulated with the highest dose of LPA. Data representing the mean [^{35}S]-GTPγS binding obtained (in dpm.) ± standard error of the mean (s.e.m.) were plotted and analysed by non-linear regression analysis with a sigmoidal dose-response curve fitting (GraphPad Prism) to allow the pEC_{50} (potency) values of LPA to be calculated in individual clones (Table 4.2).

	Basal ± S.E. *dpm*	pEC_{50} ± S.E. *concentration*	EC_{50} ± S.E. *concentration (nM)*
SK-N-SH	9107 ± 459	-7.115 ± 0.095	77 ± 30
Clone 4	8291 ± 650	-6.723 ± 0.176	190 ± 113
Clone 7	17620 ± 1643	-7.914 ± 0.245	12 ± 8
Clone 12	8463 ± 316	-7.446 ± 0.133	36 ± 17

Table 4.2. *Basal constitutive activity and LPA-induced [^{35}S]-GTPγS binding following stable transfections*

Levels of basal activity in membranes prepared from SK-N-SH cells and in *EDG2*-overexpressing clones 4, 7 and 12 are presented as dpm (disintegrations per minute) bound. Potency values of LPA calculated from [^{35}S]-GTPγS binding dose-response curves are given as pEC_{50} values. n=2 for each cell type.

Although all cells exhibited substantial basal [^{35}S]-GTPγS binding, a LPA dose-dependent increase in [^{35}S]-GTPγS binding in both wild type SK-N-SH cells and clones 4, 7 and 12 (Figure 4.10) was evident, confirming that these cells were responsive to some extent to LPA.

Basal [^{35}S]-GTPγS binding (table 4.2) displayed by wild-type SK-N-SH cells in absence of LPA and probably mediated by constitutively active GPCRs as well as activation of endogenous EDG2 and GPR23 receptors by potential residual LPA present in the assay buffer, was consistent with the literature (Yoshida and Ueda 1999), but it was more surprising that clones 4 and 12 exhibited equivalent basal [^{35}S]-GTPγS binding levels since these cells had been shown to overexpress the LPA receptor *EDG2*. Although the hypothesis that increased *EDG2* mRNA expression may be translated into functional receptors in these cells is supported, these results suggested that exogenous EDG2 receptors expressed by clones 4 and 12 did not show constitutive activity.

Conversely, clone 7 was shown to exhibit higher basal [^{35}S]-GTPγS binding than other cells suggesting that this clone expressed exogenous EDG2 receptors (Figure 4.10) that displayed an important constitutive activity.

The LPA-induced increase in [^{35}S]-GTPγS binding above basal was higher in wild-type SK-N-SH cells and *EDG2*-overexpressing clone 7 than in other *EDG2*-overexpressing clones, clone 12 appearing to be the least sensitive to LPA. Interestingly, the better sensitivity of clone 7 in this assay compared to other clones was consistent with previous results showing that this clone displayed the highest

EDG2 expression levels (section 4.2) and mediated the highest increase in JNK phosphorylation (section 4.4). Moreover, clone 7 was determined to yield the lowest pEC_{50} value of LPA (about 6 times less than that of wild-type SK-N-SH cells), further confirming the superiority of this clone for screening purposes (Table 4.2).

In conclusion, [^{35}S]-GTPγS binding assay using *EDG2*-overexpressing clone 7 was found to be a sensitive and reproducible approach that may be used for screening potential EDG2 receptor agonists.

4.7- Discussion

The aim of the work described in this chapter was 1) to characterise *in vitro* the potential role of EDG2 in relation to schizophrenia by investigating the hypothesis that stimulation of this G protein-coupled receptor may constitute a way to restore the activity of the "Pyk/Nck" pathway downstream from NMDA receptors and 2) to develop an assay which may be used for screening potential EDG2 ligand compounds.

4.7.1- Transient overexpression of *EDG2* in SK-N-SH cells and functional characterisation of the activity of the "Pyk/Nck" pathway in these cells

In order to test the hypothesis that EDG2 stimulation may constitute an alternative route to activating the "Pyk/Nck" pathway and thereby restore NMDA receptor function in schizophrenia, the *EDG2* human cDNA was cloned into an expression vector to allow overexpression of the receptor *in vitro*. pMSF1, an expression vector derived from pcDNA3.1-FLAG® whose N-terminal signal sequence helps anchoring the cloned gene into the membrane (Mitsubishi Pharma Corporation) was chosen to ensure targeting of exogenous EDG2 receptors to the plasma membrane and thereby their correct functioning as G protein-coupled receptors.

Human neuroblastoma SK-N-SH cells whose ability to proliferate and to differentiate makes an excellent system for *in vitro* studies (Shastry *et al.* 2001) were transfected with pMSF1-*EDG2* expression construct. Cells transfected with the empty pMSF1 vector were used as a negative control in subsequent functional experiments investigating the role of EDG2 in relation with the "Pyk/Nck" pathway.

Failure to confirm overexpression of the FLAG®-EDG2 fusion protein using immunodetection of pMSF1 FLAG® epitope may be explained by the presence of only 7 out of the 8 amino acids (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) of the FLAG® sequence which may be insufficient to allow specific binding of the FLAG® antibody. However, although the FLAG® antibody used (Sigma-Aldrich) was correctly detecting the 8-amino acids FLAG® epitope in similar experimental conditions (section 5.6.2), no other antibody was tried to detect pMSF1 FLAG® epitope, making it impossible to exclude that this specific FLAG® antibody may be the issue. Nevertheless, western blotting using EDG2 antibodies was attempted as an alternative strategy but despite sustained efforts to optimise the methodology, including the use of five different antibodies (section 4.2, table 4.1), this technique again failed to confirm the overexpression of EDG2.
Finally, overexpression of *EDG2* mRNA was confirmed using qRT-PCR on pMSF1-*EDG2*-transfected SK-N-SH cells. Interestingly, SK-N-SH cells were shown to express endogenous *EDG2* receptors suggesting that they may mediate some effects of LPA in these cells (Ashagbley *et al.* 1996). Although this evidence did not rule out any subsequent potential translation issues, it was indeed unlikely that the *EDG2* overexpression detected at the level of the mRNA may not result in significant overexpression of the protein.

Although transiently *EDG2*-overexpressing cells necessarily exhibited a great variability in their levels of expression of *EDG2* because of the inevitable unevenness of the transfection efficiency, they were used to investigate initially the effects of EDG2 overexpression on the activity of the "Pyk/Nck" pathway and to validate the methodology used for potential subsequent functional studies using other cells or other conditions.

Phosphorylation (hence activation) of the final output of the "Pyk/Nck" pathway, MAPK9 (JNK2), was investigated first as it was thought to reflect the activity of the signalling cascade. However, immunoblotting analysis using a phosphorylation-specific antibody was complicated by the fact that the sites of phosphorylation present in MAPK9 are actually common to all JNK isoforms (MAPK8, MAPK9 and MAPK10, or JNK1, JNK2 and JNK3 respectively) which all resolve in SDS-PA gels as either 46 or 54 kDa proteins. Thus, specifically determining which JNK isoform was potentially activated in *EDG2*-overexpressing cells was not possible.

Despite this, a dual phosphorylation (Thr183/Tyr185) specific JNK antibody was used to investigate the activation of the "Pyk/Nck" pathway in *EDG2*-overexpressing cells both in absence of LPA and after LPA treatment. As expected, the antibody detected two bands of ~46 and 54 kDa that corresponded each to a mixture of proteins, the upper band being mostly MAPK9 (JNK2) and the lower band being mostly MAPK8 (JNK1) (Mizuno *et al.* 2002).

Visual examination and ANOVA analysis of the blots measured by densitometry revealed that JNK_{46kDa} and JNK_{54kDa} followed the same activation pattern in *EDG2*-overexpressing cells and after LPA stimulation.

Thus, *EDG2* overexpression had a major effect in increasing JNK activation overall, however, the increased JNK activation observed in *EDG2*-overexpressing cells compared with cells transfected with the empty pMSF1 vector was only significant (pairwise) when the cells were stimulated with LPA suggesting that LPA binding to the overexpressed EDG2 receptors was required to induce JNK activation. Consistently, the effect of LPA treatment on JNK activation was not significant overall but in *EDG2*-overexpressing cells only.

These results provided evidence that LPA-stimulation of EDG2 receptors resulted in the similar and concomitant activation of both JNK_{46kDa} and JNK_{54kDa} proteins suggesting that EDG2 receptors overexpressed in SK-N-SH cells coupled to one or several pathway(s) leading to the activation of several MAPKs including MAPK8 (JNK1) and MAPK9 (JNK2). These results therefore confirmed the hypothesis that EDG2 overexpression and its stimulation with LPA affects the activity of the final output of the "Pyk/Nck" pathway.

However, the precise determination of which of these MAPKs were activated following LPA stimulation of EDG2 receptors may be very important functionally. JNK proteins have been shown to display differing affinities and specificities for target transcription factors so that they may induce the activation of distinct pathways and cellular responses (Bozyczko-Coyne *et al.* 2002). However, even though antibodies exist which make it feasible to resolve activation of JNK family subgroups by immune-complex and/or immuno-depletion kinase assays, these assays are not straightforward. Thus, as an attempt to specifically investigate the activation of MAPK9 in *EDG2*-overexpressing cells, a JNK activation assay was developed coupling immunoprecipitation with a phospho-specific JNK (JNK-P) antibody (detecting the active form of JNK) with immunodetection (western blotting) using a JNK2 antibody. As expected, this assay specifically detected phosphorylated MAPK9 (JNK2) proteins of approximately 54 kDa but it proved quantitatively non-reproducible, which was unacceptable since the purpose of this assay was to investigate semi-quantitatively the activation of MAPK9 in *EDG2*-overexpressing cells.

Another technique for profiling the MAPK pathway downstream of EDG2 receptors would have been to study the activation of signal transduction by quantitatively measuring transcription activation but this method, which would have necessitated further cloning experiments to set up reporters vectors, would have investigated downstream events and therefore would not have addressed the hypothesis of the "Pyk/Nck" pathway and MAPK9 being downstream of EDG2 receptors.

In order to provide confirmation of the JNK-P western blot results, a JNK-P ELISA assay was used as a semi-quantitatively more reliable method for measuring JNK phosphorylation. Use of this assay was also encouraged by results showing similar activation of both JNK_{46kDa} and JNK_{54kDa} rather than specific activation of a specific JNK isoform in LPA-stimulated *EDG2*-overexpressing cells. Thus, this assay, which did not have the power to discriminate between JNK isoforms, appeared an easier, quicker and more reliable way of assessing MAPK activity in *EDG2*-overexpressing cells.

Very interestingly, statistical analysis of JNK-P ELISA data proved exactly identical to the analysis of western blot data, showing that *EDG2* overexpression in SK-N-SH

cells induced an overall increase in JNK phosphorylation which, however, was only statistically significant after LPA treatment when comparing *EDG2*-overexpressing cells to cells transfected with the empty pMSF1 vector. Moreover, still consistent with western blotting results, LPA treatment significantly increased JNK phosphorylation in *EDG2*-overexpressing cells only, suggesting that LPA stimulation of exogenous *EDG2* receptors is coupled to a signalling pathway leading to JNK activation.

The only apparent difference between JNK-P western blots and ELISA results was in the endogenous levels of JNK phosphorylation measured by the ELISA assay in non-transfected SK-N-SH cells compared with western blotting. This difference may be explained by the better sensitivity of the ELISA technique compared to western blot analysis, especially since less total protein was used for the ELISA (15μg) than for western blotting (40μg).

In conclusion, these results provided evidence that LPA-stimulation of EDG2 receptors induced the activation of the final output of the "Pyk/Nck" signalling pathway, MAPK9, probably along with other MAPK. Whether the signal induced by LPA stimulation was actually transduced via the "Pyk/Nck" pathway may be determined by looking at the activation of other proteins within the hypothesised cascade (section 4.5). The similar activation pattern of JNK_{46kDa} and JNK_{54kDa} induced by LPA in *EDG2*-overexpressing SK-N-SH cells suggested that EDG2 receptors stimulated by their endogenous ligand probably coupled to multiple signalling pathways leading to various responses in terms of transcription activation. This hypothesis was consistent with evidence showing that LPA can mediate several intracellular transduction pathways via activation of different G proteins (Moolenaar *et al.* 1997; Yoshida and Ueda 1999; Yoshida and Ueda 2001).

However, this did not rule out the possibility that EDG2 receptors may potentially possess agonists or may be stimulated by synthetic ligands mediating the activation of selected transduction pathways hence potentially specific MAPK. Such a mechanism, shown for lysophosphatidylcholine (LPC) and sphingosine 1-phosphate (S1P) lipid receptors may indeed be envisaged for LPA EDG2 receptors, which are phylogenetically very close to LPC and S1P receptors (Uhlenbrock *et al.* 2002; Lin and Ye 2003).

These preliminary functional studies performed using transient *EDG2*-overexpressing cells therefore provided promising evidence suggesting that EDG2 stimulation by LPA may mediate the activation of a signalling pathway leading to MAPK activation. However, LPA-induced activation of MAPK has been shown to be highly dependent on the cell phenotype with different protein-tyrosine kinases (including Ptk2b) mediating signal transduction (Buist *et al.* 1998) suggesting that it would be very premature to translate directly these results to any hypothesis *in vivo*. Moreover, the massive overexpression of exogenous *EDG2* receptors (approximately 1.75×10^5 times more than wild-type SK-N-SH cells) in these cells may affect the pharmacology of EDG2 receptors (Kenakin 2003) so that these results may not reflect the physiology of endogenous neuronal EDG2 receptors *in vivo*. These issues are discussed in more detail in section 4.7.5.

4.7.2- Development of a stable transformed *EDG2*-overexpressing cell line and functional characterisation of the activity of the "Pyk/Nck" pathway in these cells

Promising functional results showing activation of MAPK9 after LPA stimulation of EDG2 receptors in transiently *EDG2*-overexpressing cells (section 4.3) encouraged the generation of a stable transformed cell line for further functional studies on the "Pyk/Nck" pathway and potentially to develop an assay for screening EDG2 ligand compounds. Having a constant phenotype is indeed essential to obtain reliable and reproducible results from *in vitro* functional studies and screening assays, for which a large number of clonal cells is usually needed (Eglen 2005).

Creation of a stable cell line overexpressing EDG2 receptors was also stimulated by promising *in vivo* data obtained concomitantly in the lab showing that acute Edg2 activation restores schizophreniform sensorimotor gating deficits in prepulse inhibition (PPI) (Alice Egerton) and reverses PCP-induced deficits in prefrontal cortex metabolic hypofunction (Susan Cochran and Allan McVie). These results suggesting that Edg2 agonists may have an antipsychotic activity provided further evidence that EDG2 may potentially represent a promising drug target against schizophrenia and reinforced the need for an *EDG2*-overexpressing cell line of constant phenotype that may be used in a potential screening assay.

An *EDG2*-overexpressing stable cell line was thus developed from transiently *EDG2*-overexpressing SK-N-SH cells. After a thorough selection process, 15 clones were obtained and three of them were found to show significantly higher *EDG2* mRNA expression levels than wild-type SK-N-SH cells. One clone in particular, clone 7, exhibited the largest *EDG2* expression, which was lower than that of transiently EDG2-overexpressing cells.

In order to provide further confirmation of previous results showing coupling of the EDG2 receptors to MAPK, these cells were used in the JNK-P ELISA assay, a more effective and sensitive method to semi-quantitatively detect JNK activation (section 4.3.2). Stable *EDG2* overexpression induced JNK activation which was consistent with previous results using transiently *EDG2*-overexpressing cells. Moreover, maximal JNK activation was observed in *EDG2*-overexpressing clone 7 which interestingly corresponded to the highest expression levels of *EDG2* mRNA in this clone compared to clones 4 and 12 (section 4.2). However, LPA treatment, which increased JNK activation in transient *EDG2*-overexpressing cells, showed the opposite effect in stable *EDG2*-overexpressing clones, significantly decreasing JNK phosphorylation in clones 4 and 12 (section 4.4). LPA-induced decrease in JNK activation was not significant in clone 7, which may have suggested that this clone was superior to other clones in terms of specificity of transduced signalling pathways. However, although this possibility could not be excluded, the apparent decrease in JNK activation induced by LPA in this clone similar to other clones rather suggested that all three clones were undergoing the same mechanism when stimulated by LPA.

Thus, stable overexpression of *EDG2* in these cells in the absence of LPA may induce conformational changes in the GPCRs, favouring a constitutively active state in which the receptors may be able to initiate a biochemical response in the absence of LPA, as opposed to a state in which they are activated by their ligand, LPA. Ligand independent signalling has been reported for many GPCRs (Seifert and Wenzel-Seifert 2002) including receptors phylogenetically close to EDG2 (Kostenis 2004) such as the EDG S1P receptors (Lee *et al.* 1996; Niedernberg *et al.* 2003) and the non-EDG cannabinoid and melanocortin receptors (Nie and Lewis 2001; Holst and Schwartz 2003) and is specifically a very common feature displayed by

transformed cell lines (Seifert and Wenzel-Seifert 2002). Following this hypothesis, LPA stimulation of constitutively active EDG2 receptors may induce a change in the conformation of the receptors towards a ligand-activated state (Gether and Kobilka 1998). However, this LPA-induced effect may be progressive and dose-dependent so that it may be necessary to extend the duration of LPA treatment or delay before investigation in order to reveal the maximal activation of LPA-induced downstream signalling pathways. Thus, the duration of LPA treatment or the time-point used to look at LPA-induced JNK activation in these stable transformed cells may not be long enough to enable most of the receptors to switch conformation and become activated by LPA, so that the genuine effect of LPA in mediating activation of the MAPK signalling pathway may be masked under these conditions. Moreover, since the JNK-P ELISA assay cannot differentiate between JNKs, it may be that constitutively active receptors and LPA-activated EDG2 receptors actually couple to different JNK pathways, a mechanism which may be masked in the ELISA assay quantifying the whole phosphorylated JNK proteins. Such a phenomenon has indeed been shown for other receptors including dopamine D2 and opioid receptors (Kenakin 2003).

Although it may not be as likely, another possibility which may explain the decrease in JNK activation induced by LPA treatment in stable *EDG2*-overexpressing cells may be the desensitisation of these receptors (Ferguson *et al.* 1996; Conway *et al.* 1999). LPA-induced internalisation of EDG2 receptors resulting in decreased responsiveness to their ligand has indeed been shown to participate in the regulation of the activity of EDG2 (Avendano-Vazquez *et al.* 2005) and EDG1 (Liu *et al.* 1999) receptors and may explain the limited activation of JNK observed in stable *EDG2*-overexpressing cells after LPA treatment.

Again, in order to address these issues, it may be very interesting to investigate the activation of specific JNK pathways in stable *EDG2*-overexpressing cells in the absence and in presence of LPA.

Although levels of JNK activation were decreased in LPA-treated cells compared to cells treated with a vehicle, they were still massively higher in LPA-stimulated *EDG2*-overexpressing clones, especially clone 7, than those displayed by wild-type SK-N-SH cells, suggesting that stable exogenous EDG2 receptors mediated the activation of (a) signalling pathway(s) leading to MAPK phosphorylation. Of three

stable *EDG2*-overexpressing clones, clone 7 appeared the most promising to functionally investigate the "Pyk/Nck" pathway and to be used in potential screening purposes.

In order to investigate whether stimulation of EDG2 receptors leading to MAPK phosphorylation was mediated by the "Pyk/Nck" pathway, and thereby test the hypothesis that EDG2 stimulation may provide another route to activating this pathway, potential gene expression changes of several components of this cascade, including *NCK1*, *PAK1* (Nck1-interacting protein), *PTK2B* and *MAPK9*, were evaluated using the stable *EDG2*-overexpressing clones. Because like most signalling cascades, the "Pyk/Nck" pathway is a series of kinases whose subsequent phosphorylation or dephosphorylation allows quick transduction of the signal, the methodology (qRT-PCR) as well as the design (absence of LPA treatment) of this study may not be ideal to investigate the activity of the "Pyk/Nck" pathway but may nevertheless provide an insight on the genes/proteins that may be regulated by EDG2 signalling.

Most of the kinases examined including *PTK2B*, *MAPK9*, and *PAK1* did not exhibit any change in expression in *EDG2*-overexpressing cells compared with wild-type SK-N-SH cells (section 4.5) which was consistent with the hypothesis that activation of these proteins occurs by phosphorylation rather than regulation of gene expression. Thus, unchanged expression of *MAPK9* in stable *EDG2*-overexpressing cells was consistent with JNK-P ELISA results showing an increased MAPK phosphorylation in these cells by comparison with wild-type SK-N-SH cells (section 4.4). Nevertheless, one of the genes examined, *NCK1*, an adaptor protein shown to regulate JNK activation (Mizuno *et al.* 2002; Poitras *et al.* 2003), showed decreased expression in stable *EDG2*-overexpressing cells suggesting that EDG2 receptors did mediate MAPK activation via the "Pyk/Nck" signalling cascade. Interestingly, this result was consistent with data showing a massive increase in MAPK activation in the prefrontal cortex of *Nck1* knock-out mice (Nicola Veitch).

In conclusion, these results provided further evidence that EDG2 receptors mediate the activation of the "Pyk/Nck" pathway leading to MAPK activation so that stimulation of these receptors may represent a therapeutic strategy to counterbalance PCP-induced deficits within this pathway and thereby its behavioural correlates.

4.7.3- [^{35}S]-GTPγS binding assay on EDG2 clones

In order to allow screening of potential EDG2 agonists in vitro, a [^{35}S]-GTPγS binding assay was developed using stable *EDG2*-overexpressing cells (section 4.6). This assay, which monitors guanine nucleotide exchange at G-proteins upon agonist stimulation, was preferred to radioisotopic labelling of LPA because of its power to differentiate between agonists and antagonists and to determine their pharmacological parameters (Eglen 2005). Since this assay was specific to all LPA-stimulated GPCRs rather than to EDG2 receptors, *EDG2*-overexpressing SK-N-SH cells were assayed for expression of other LPA receptors including *EDG4*, and *EDG7* and *GPR23* using qRT-PCR. Lack of expression of *EDG4* and *EDG7* and very low endogenous levels of *GPR23* in these cells confirmed that a LPA-stimulated [^{35}S]-GTPγS binding assay using these cells would be quite specific to EDG2 receptors.

Wild-type SK-N-SH cells and *EDG2*-overexpressing clones exhibited basal [^{35}S]-GTPγS binding but LPA treatment dose-dependently increased [^{35}S]-GTPγS binding in all cells confirming that they were responsive to LPA. The highest basal [^{35}S]-GTPγS binding levels displayed by *EDG2*-overexpressing clone 7 compared to clones 4 and 12 and wild-type SK-N-SH cells suggested that the exogenous EDG2 receptors expressed in this clone possessed an important constitutive activity which was consistent with the highest JNK activation exhibited by this clone in absence of LPA (section 4.4). In this way, further extrapolation of JNK activation data to [^{35}S]-GTPγS binding may suggest that clones 4 and 12 would display lower basal [^{35}S]-GTPγS binding, but still higher than wild-type SK-N-SH cells. Conversely, the similarity between the [^{35}S]-GTPγS binding levels displayed by all these cells indicated that the exogenous EDG2 receptors expressed by clones 4 and 12 did not show constitutive activity. Indeed, it would be very unlikely that increased *EDG2* mRNA expression (section 4.2) would only be translated into protein in clone 7 but not clones 4 and 12 especially since they were shown to display increased JNK activation compared to wild-type SK-N-SH cells (section 4.4). Therefore the levels of EDG2 expression in clones 4 and 12 may be below the threshold required to show elevated basal [^{35}S]-GTPγS binding.

LPA dose-dependently increased [^{35}S]-GTPγS binding in *EDG2*-overexpressing clones as well as wild-type SK-N-SH cells, clone 12 being the least sensitive to LPA. While clone 4 appeared intermediately sensitive to LPA, *EDG2*-overexpressing clone 7 and wild-type SK-N-SH cells seemed about equivalently responsive to LPA, showing apparently parallel [^{35}S]-GTPγS binding dose-response curves (section 4.6, figure 4.10). However, the potency of LPA (effective concentration 50%: EC_{50}) in these cells revealed that *EDG2*-overexpressing clone 7 was actually more sensitive to LPA than wild-type SK-N-SH cells (table 4.2). Stable *EDG2* overexpression in clone 7 did induce shifting of the concentration of LPA giving 50% of maximal [^{35}S]-GTPγS binding (EC_{50}) by a factor of 6 compared to wild-type SK-N-SH cells which reflected a major improvement in the sensitivity of the assay in detecting agonist-induced [^{35}S]-GTPγS binding. Interestingly, these results using SK-N-SH cells were consistent in terms of dose-response curve with [^{35}S]-GTPγS binding results on EDG2 receptors performed using other cell lines (McAllister *et al.* 2000). Moreover, the EC_{50} of LPA using clone 7 (12nM) was comparable with those found both in a similar assay used for characterising binding of novel potential agonists to the EDG2, EDG4 and EDG7 receptors (EC_{50} of LPA: 17nM) (Santos *et al.* 2004) and in a serum response element-driven luciferase expression reporter gene assay (EC_{50} of LPA: 10nM) (An *et al.* 1998). Thus, clone 7 appeared superior to other *EDG2*-overexpressing clones and to wild-type SK-N-SH cells in the characterisation of EDG2 agonist binding.

Nevertheless, the high levels of basal [^{35}S]-GTPγS binding displayed by this clone and to a less extent by other clones and wild-type SK-N-SH cells may be pointed out as a caveat of this experiment since it may prevent the proper detection of LPA-induced [^{35}S]-GTPγS binding. Thus, even if non-specific binding was not assessed either in similar studies on other EDG receptors such as EDG1 (Parrill *et al.* 2000) and EDG7 (Im *et al.* 2000), use of unlabelled or "cold" GTPγS to ensure blockade of all potential non-specific sites before introduction of [^{35}S]-GTPγS may have been advantageous in this assay. Moreover, since binding of [^{35}S]-GTPγS to G-protein subunits *in vitro* has been shown to be highly dependent on the concentration of added GDP (Harrison and Traynor 2003), increasing the concentration of GDP in the assay buffer may also be advantageous to reduce basal [^{35}S]-GTPγS binding but

since GDP competes [^{35}S]-GTPγS for binding, it would probably reduce the rate and the magnitude of LPA-induced [^{35}S]-GTPγS binding as well and thereby may not improve the sensitivity of the assay. Moreover, the concentration (10μM) of GDP used in this study was similar to that used for the characterisation of novel Edg2 agonists *in vitro* using other cells (Santos *et al.* 2004) suggesting that this concentration was suitable for screening purposes.

Finally, in order to overcome the issue of basal [^{35}S]-GTPγS binding, it may have been advantageous to assess the expression of LPA receptors in several cell lines before using SK-N-SH cells. Different cell lines have indeed been found to show different basal and LPA-induced [^{35}S]-GTPγS binding responses (McAllister *et al.* 2000) probably depending on their endogenous expression of LPA EDG2, EDG4, EDG7 and GPR23 receptors. Since LPA, the simplest of all glycerophospholipids, is found in 2-20μM concentrations in mammalian serum (Moolenaar 1995), any expressed LPA receptors may mediate the effects of LPA in cell-based assays which may complicate the interpretation of GPCR selectivity studies (McAllister *et al.* 2000). To overcome this issue, use of pertussis toxin-insensitive EDG2 receptor-Giα1 fusion protein has been proposed as an approach to monitor EDG2 receptor-mediated (particularly Gi-mediated) responses *in vitro* (McAllister *et al.* 2000). Moreover, this technique not only improves sensitivity of the assay but, similar to methods involving immunoprecipitation or immobilisation of the G-[^{35}S]-GTPγS complexes after the assay prior to measuring the degree of radioactivity (Harrison and Traynor 2003; Eglen 2005), it enables the measurement of [^{35}S]-GTPγS binding to specific G-subunit families.

All these techniques may be successfully applied to the EDG2 [^{35}S]-GTPγS binding assay and may improve its sensitivity. Nevertheless, since the [^{35}S]-GTPγS binding assay using clone 7 was found to be as sensitive as the assay used by Santos *et al.* to characterise potential novel EDG2 agonists (Santos *et al.* 2004), this assay appeared sensitive enough to use as a screening assay and therefore suitable for this purpose.

4.7.4- Interpretation of EDG2 functional studies in relation with the "Pyk/Nck" pathway hypothesis

Evidence from *in vitro* functional studies (JNK-P ELISA and qRT-PCR) using stable *EDG2*-overexpressing cells provided strong evidence that LPA stimulation of stably overexpressed EDG2 receptors mediated the activation of a signalling pathway leading to MAPK9/MAPK10 in SK-N-SH cells. Differential expression of *NCK1* in these EDG2-overexpressing cells suggested that EDG2-stimulated increase in MAPK phosphorylation was mediated by the "Pyk/Nck" pathway.

Moreover, correlation between JNK-P ELISA and [^{35}S]-GTPγS binding results using stable *EDG2*-overexpressing cells suggested that *EDG2*-overexpressing clone 7 was superior to other transformed clones in mediating activation of the "Pyk/Nck" pathway. This clone, which may be suitable for use in [^{35}S]-GTPγS binding assay to screen potential EDG2 agonists, may also be advantageously used in further functional studies that may address the specificity of the MAPK pathway activated downstream of EDG2 receptors in SK-N-SH cells in response to stimulation by LPA and/or by potential EDG2 agonists. Such studies may include the investigation of potential changes in the activation, through phosphorylation, of "Pyk/Nck" pathway proteins, using western blots or ELISAs, and the examination of EDG2 downstream "Pyk/Nck" signalling using *EDG2*-overexpressing cells transfected with dominant negative constructs of specific "Pyk/Nck" genes.

As for most functional studies and screening assays for G-protein coupled receptors, a recombinant cell line overexpressing *EDG2* receptors was used to investigate the activation of signalling pathways mediated by these receptors following stimulation with their endogenous ligand, LPA. SK-N-SH neuroblastoma cells were selected to develop this recombinant cell line because these human cells commonly used in neurobiology could potentially be differentiated into cells of neuronal-like phenotype (Shastry *et al.* 2001). Nevertheless, however suitable these cells appeared for our studies, their background (similar to that of any cells) necessarily limited our results to their phenotype (Kenakin 2003). GPCR pharmacology has indeed been shown to be very specific to the cell type which influences the formation of GPCR oligomers and affects the action of allosteric ligands (Eglen 2005). Moreover, the level and pharmacology of GPCR is determined by the cell-specific nature and expression of

GPCR-interacting proteins (GIPs) (Eglen 2005). Thus, both the relative potency of compounds and G-protein selectivity is very specific to the cell type used (Kenakin 2003). With regards to LPA for example, LPA-stimulated activation of MAPK has been shown to be mediated by different pathways and interacting proteins in COS-7, PC12 cells and fibroblasts (Buist *et al.* 1998). In SK-N-MC cells neuroepithelioma cells, which are phenotypically very similar to SK-N-SH cells, LPA-induced MAPK activation was not confirmed (Buist *et al.* 1998). However since the kinase assay used in this study only detected p42 MAPK activity these results do not contradict evidence showing LPA-induced activation of JNK MAPK in SK-N-SH cells (sections 4.7.2 and 4.7.3).

Moreover, although recombinant cell lines are a very valuable tool in drug discovery, they do not reproduce the physiological conditions and signal transduction from receptor to G-protein using such cells may not be as efficient and selective as when all signalling components have endogenous expression levels (Kurkinen *et al.* 1997). Each G-protein has indeed a distinct affinity for the receptor so that changes in receptor density due to massive overexpression of GPCRs may induce differences in the quality (composition of signals) as well as the quantity of response to agonists (Kenakin 2003).

Early models of GPCR function comprising two receptor states (active and inactive) are therefore widely obsolete especially since incoming signals from a ligand do not produce a uniform output signal from the receptor but can produce a signal distributed to various pathways (Kenakin 2003). Thus, it is likely that, as has been shown for many receptors, EDG2 receptors may be stimulated by different agonists activating specific cellular pathways, a phenomenon referred to as stimulus trafficking (Kenakin 1995). Moreover, studies have also indicated that the receptor active state mediating constitutive activity differs from the state created by agonist binding which may explain the decrease in JNK-P activation observed in stable *EDG2*-overexpressing cells in response to LPA (section 4.4).

These data therefore further reinforce the need and the interest of investigating LPA- and agonist-induced activation of EDG2 receptors and their downstream signalling pathways. In particular, it may be advantageous to characterise the stimulation of EDG2 by the LPA derivatives used *in vivo* as EDG2 agonists (Santos *et al.* 2004) in terms of [^{35}S]-GTPγS binding and mediated signalling pathways.

Beyond these considerations, these *in vitro* results provided evidence that EDG2 stimulation may constitute a novel route leading from activation of the "Pyk/Nck" signalling cascade down to MAPK activation. How the activation of this pathway and of its final output JNK, relates to schizophrenia remains a very difficult question because of the complexity of the JNK signalling pathway and of the potential diversity of its functions depending on environmental factors and different cell types (Davis 2000; Johnson and Lapadat 2002; Vlahopoulos and Zoumpourlis 2004; Waetzig and Herdegen 2004). Nevertheless, recent evidence of the physiological functions of JNK in the development of the brain and the integrity of the cytoskeleton appear particularly interesting since they may relate NMDA downstream signalling events with the neurodevelopmental hypotheses of schizophrenia (Lewis and Levitt 2002), while the roles of JNK in synaptic plasticity may be consistent with the hypothesis of altered synaptic plasticity and connectivity in schizophrenia (Stephan *et al.* 2006). Consistent with literature suggesting a role for EDG2 in myelination (Contos *et al.* 2000b; Handford *et al.* 2001), coupling of this receptor to the "Pyk/Nck" signalling cascade may therefore provide an example connecting a differentially expressed receptor with several hypotheses including the emerging neurodevelopmental hypothesis of schizophrenia (Rapoport *et al.* 2005).

In the light of the deficits in the activity of this pathway downstream of NMDA receptor signalling, modulating EDG2 activity may therefore represent a novel strategy to counterbalance PCP-induced deficits in the rat chronic PCP model and NMDA receptor hypofunction in schizophrenic patients. Interestingly, further support to this hypothesis was obtained *in vivo*, with evidence showing that acute Edg2 activation using a novel Edg2 agonist developed by MPC restores schizophreniform sensorimotor gating deficits in prepulse inhibition (PPI) in mice (Alice Egerton) and reverses PCP-induced prefrontal cortex metabolic hypofunction (Susan Cochran and Allan McVie). These results suggested that EDG2 agonists may have an antipsychotic activity, strongly reinforcing the interest in EDG2 as a potential promising drug target for schizophrenia.

CHAPTER 5: FROM THE EST AI072720 TO AN UNCHARACTERISED GENE POTENTIALLY IMPLICATED IN SCHIZOPHRENIA

5.1- Introduction

The aim of this work was to confirm the EST AI07270 as a genuine rat transcript and to further characterise it in relation with schizophrenia. Transcript analysis methods (RACE and RT-PCR) were used for identifying the rat gene and confirming its differential expression in the chronic PCP model of schizophrenia. To investigate its function, a variety of cellular biology techniques were used, including cloning, overexpression and immunofluorescence to look at subcellular localisation and to examine cell morphology *in vitro* and immunoprecipitation for identifying interacting proteins. The effect of KIAA1189 (approved name of human orthologue) overexpression on neurite outgrowth of PC12 cells was also examined. Genotyping of single nucleotide polymorphisms (SNPs) was finally used in order to explore any potential genetic association of this gene with schizophrenia in a Caucasian population of schizophrenic patients and controls.

5.2- *In silico* analyses towards the identification of a gene for the EST AI072720

5.2.1- DNA sequence homology screening

Initial DNA sequence homology screening analyses (section 2.3.2, November-December 2003) showed that the human orthologous region of rat EST AI072720 did indeed correspond to the 3' untranslated region (3'UTR) of a then predicted and now approved gene called *KIAA1189* (71% identity over 140bp of the Affymetrix rat probe set target sequence and human cDNA sequence). No homologous mouse or rat gene was found at Ensembl but interestingly the Affymetrix rat probe set target sequence showed strong sequence identity (99.5% over 386bp) with a rat genomic sequence (Chr.3 39717303-39717688) located 2063bp downstream of the last exon of a predicted rat gene now called *RGD1308367*, orthologous to the human *KIAA1189* gene.

This suggested that the EST AI072720 may belong to a part of the rat orthologue of human *KIAA1189* which had not been cloned or predicted yet. We hypothesised that

despite this EST sequence being 2kb downstream of *RGD1308367*, it may represent a splice variant or an alternative exon of this gene or belong to its 3' untranslated region (3'UTR) (figure 5.1).

Alternatively, the EST AI072720 could represent a novel rat gene, not cloned or predicted.

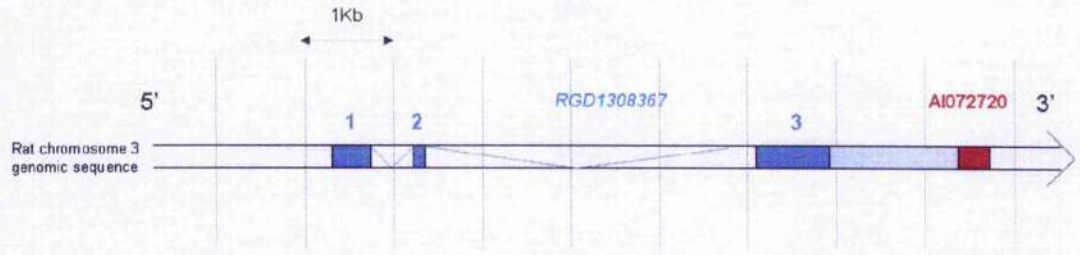


Figure 5.1. *Locations of EST AI072720 and RGD1308367 (KIAA1189 orthologue) within the rat genomic sequence*

Blue boxes represent exons 1, 2 and 3 of predicted rat RGD1308367 gene, red box represents the EST and white regions represent intronic or intergenic genomic DNA. The pale blue box shows the location of EST AI072720 in relation with exon 3 of *RGD1308367*. *RGD1308367* is the current and approved symbol of the rat gene named "*similar to KIAA1189*" which is the rat orthologue of human *KIAA1189* (approved gene name).

5.2.2- Sequence information retrieval and clustering of EST

Clustering and assembling ESTs is a valuable and effective method to reconstruct transcripts and discover new genes or new variants of genes.

In order to correlate and associate the EST AI072720 with other potential overlapping or neighbouring ESTs, the EST AI072720 was mined from different databases, including GenBank (nucleotide database including dbEST for information on ESTs), Unigene (gene-oriented clusters of transcript sequences), UniSTS (markers and mapping data) and Geo Profiles (expression and molecular abundance profiles) databases (all at NCBI).

A description of the source of the EST was found in the GenBank/dbEST annotation for AI072720: the EST AI072720 (Rn#S13892600, mRNA reference gi|3398914) was identified as a clone in a subtracted cDNA library prepared from an adult rat

individually-tagged normalised whole-eye (minus the lens) library (Bonaldo *et al.* 1996)

At Unigene, the EST AI072720 was found to belong to a cluster of 4 ESTs (Unigene cluster Rn.20243) mapped to rat chromosome 3 (UniSTS entry: chr.3 RH134705). Interestingly, the 3 other ESTs of this cluster were identified as clones in libraries derived from brain tissue only:

- BF568003 (Rn#S14101815, 5' read) was cloned from dbEST3768, a subtracted library derived from a mixture of the following tissues: thalamus, cerebellum, hypothalamus, medulla, pons, midbrain, cerebral cortex, corpus striatum and hippocampus,
- CB734608 (Rn#S15097956, 5' read) was cloned from dbEST12821, a Wistar rat hypothalamus and pituitary gland library,
- CB781779 (Rn#S15141528, 3' read) was cloned from dbEST12817, a rat hypothalamus library.

No Geo Profile reporting the identification of the EST AI072720 from microarray studies was found.

ClustalW at EBI-EMBL showed a very good alignment and a high similarity between the four Rn.20243 cluster ESTs sequences (including EST AI072720). A consensus overlapping sequence (common between all 4 sequences) of about 320bp was identified and the EST AI072720 (Rn#S13892600) was found to exhibit 86.2% sequence identity over 807bp with the EST Rn#S14101815. Among the 4 sequences, Rn#S14101815 was found to be the longest extending at the 5' and 3' ends. The alignments did not show 100% sequence identity between the ESTs, probably because of sequencing errors. However the alignment of 4 EST sequences added weight to the hypothesis that the EST AI072720 was a genuine rat transcript.

5.2.3- Gene prediction

Many gene prediction programs based on different methods and algorithms of gene prediction were used to identify a gene for the EST AI072720.

In order to maximise the chance both to confirm that the EST AI072720 belonged to *RGD1308367*, the rat orthologue of *KIAA1189* gene, or to predict a novel gene in which the EST AI072720 was contained, these programs were run on a 7282bp-long

sequence corresponding to the EST AI072720 sequence extended with 5' genomic sequence (Chr.3 39717251-39724532, minus strand orientation).

Among the programs run, Genscan, HMMGene, EUI and GI (within GeneComber) predicted the three exons of the upstream *RGD1308367* gene but did not predict anything in the region of the EST AI072720. Figure 5.2 shows the Genscan output as an example.

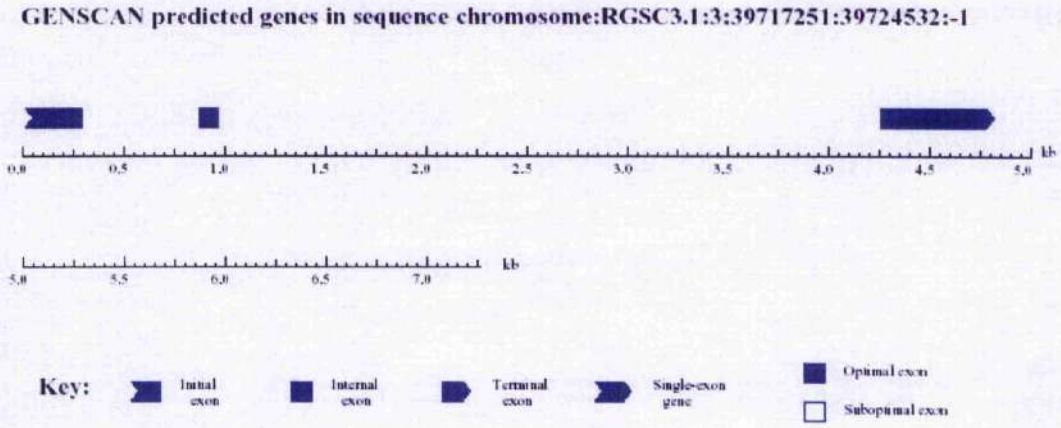


Figure 5.2. *Genscan graphical output for rat chromosome 3 39717251-39724532 (minus strand)*

The scale bar represents genomic distance in kb. The blue blocks represent the 3 predicted exons upstream of EST AI072720.

In contrast, GRAIL, GeneID, the Integrated system for exon finding (MZEF + SPC), the ORNL Genome Analysis Pipeline and NIX all predicted four exons within the 7.3Kb sequence.

GRAIL and The Integrated system for exon finding predicted, in addition to the three exons of *RGD1308367*, an exon of 135bp from 6754 to 6889 (with regards to the entry sequence, 39717643 to 39717778 with regards to rat genomic sequence). Interestingly, this putative fourth exon was found to align exactly with 41bp at the 5' end of the EST AI072720 sequence (Affymetrix consensus sequence) and its sequence was shown to be part of the sequence of the EST Rn#S14101815, *i.e.* the longest of the 4 members of rat cluster Rn.20243.

GeneID predicted many several possible fourth exons whose sequence also overlapped with the AI072720 sequence.

In the context of the ORNL Genome Analysis Pipeline, GrailEXP also predicted a fourth exon starting in position 6662 (rat chromosome 3 39717870) that again

overlapped with the EST AI072720 sequence. Moreover, post-processing analysis of this transcript by the ORNL Analysis Pipeline predicted a putative protein that may contain a Pfam C-terminal Ezrin/Radixin/Moesin (ERM) domain in the third exon, consistent with predictions on both rat *RGD1308367* and human *KIAA1189* genes at Ensembl.

Finally, NIX was used as a tool for running additional programs on the 7.3kb entry sequence. Grail, Genscan, HMMGene and MZEF gave the same results as when they had been run separately. Among the other programs NIX includes, GeneMark very interestingly identified a "region of interest" from 6659 to 6913 of the entry sequence (rat chr.3 39717619 to 39717873) and predicted a "protein-coding exon" from 6729 to 6816 (rat chr.3 39717716 to 39717803), *i.e.* about 30bp upstream from the EST AI072720.

In conclusion, some gene prediction programs did predict the presence of a piece of transcript from the genomic sequence located around the EST AI072720 but some others did not. Although this evidence was not sufficient to draw any definite conclusion, it suggested that the EST was likely to be part of an uncloned and uncharacterised region of *RGD1308367*.

Brain-specific expression of the ESTs of the AI072720 cluster together with confirmation of the differential expression of EST AI072720 after chronic PCP treatment (section 3.4.3) provided evidence of a potential role of this gene in brain processes in general and potentially in prefrontal cortex dysfunction in schizophrenia.

5.3- From EST AI072720 to *RGD1308367* ("*similar to KIAA1189 protein*") gene: RACE PCR, RT-PCR

RACE (Rapid Amplification of cDNA ends) PCR was used to investigate the expression of the EST AI072720 in the rat brain as part of a larger transcript and in particular as part of the upstream predicted *RGD1308367* gene.

Prior to performing the 5' RACE PCR, the expression of EST AI072720 was confirmed in the rat brain cDNA library (Clontech) that was to be used for the RACE. PCR using both qRT-PCR forward and reverse primers (Figure 5.3) was performed and a discrete band of the expected molecular weight was obtained (data not shown).

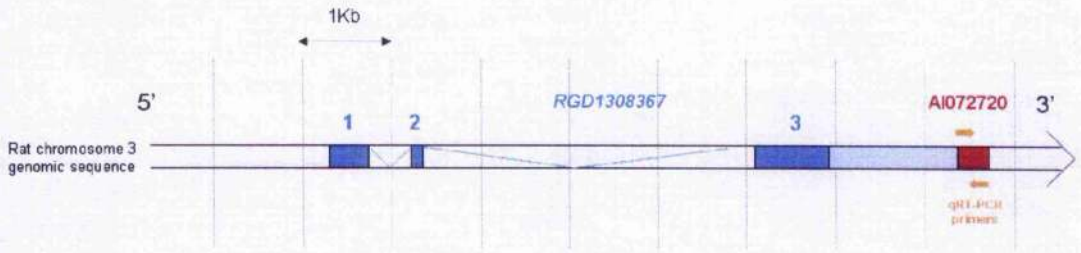


Figure 5.3. *Positions of qRT-PCR primers with respect to AI072720 and RGD1308367*

This figure shows the respective positions of the forward and reverse qRT-PCR primers which were used to amplify EST AI072720.

5' RACE PCR was performed on rat brain cDNA using an adaptor specific primer and a primer specific to EST AI072720 that was based on the qRT-PCR reverse primer. Figure 5.4 shows the cDNA template and the position of the primers used in the 5'RACE PCR.

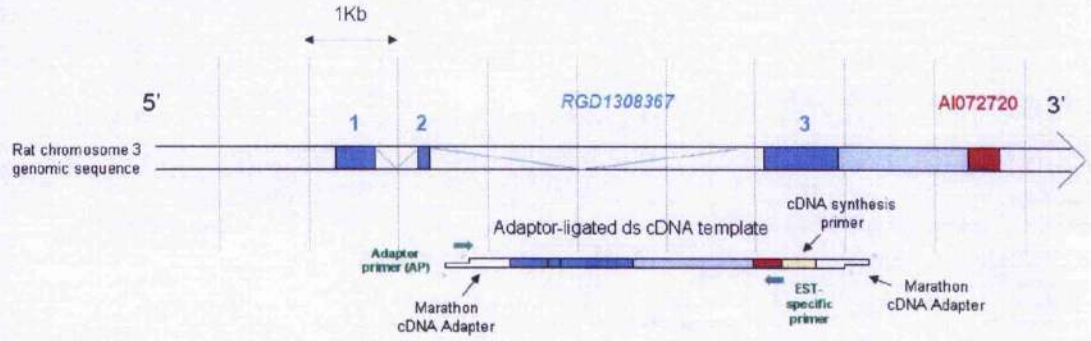


Figure 5.4. *cDNA template and primers used in the 5'RACE PCR with respect to AI072720 and RGD1308367*

Marathon-Ready cDNAs (Clontech), whose structure is depicted above in the context of the amplification of *RGD1308367*, is essentially a library of uncloned ds cDNA that allows the amplification of specific cDNAs using different sets of primers. Within the adaptor-ligated ds cDNA template, marathon cDNA adapters are represented in white while the primer used for synthesising the cDNA is shown in yellow. In order to amplify the sequence located upstream of EST AI072720, a reverse EST-specific primer was used in combination with a forward adaptor-specific primer (Clontech) whose sequence was based on the cDNA adaptor ligated.

5'RACE PCR resulted in the amplification of a 3.4kb product as well as a lower molecular weight smear (Figure 5.5, lane 1).

The control reactions gave expected results:

- the *GAPDH* 5' RACE positive control (lane 2) generated a product of about 1kb (expected product is 1.09kb long according to Clontech) together with an unexpected product of 700bp and a smear of products,
- PCR using the RACE EST-specific primer and the forward primer used in qRT-PCR (positive control, lane 3) generated a product shorter than 100bp (expected size of amplicon: 80bp),
- negative controls without any cDNA (lanes 4, 5 and 6) did not show any amplified product.

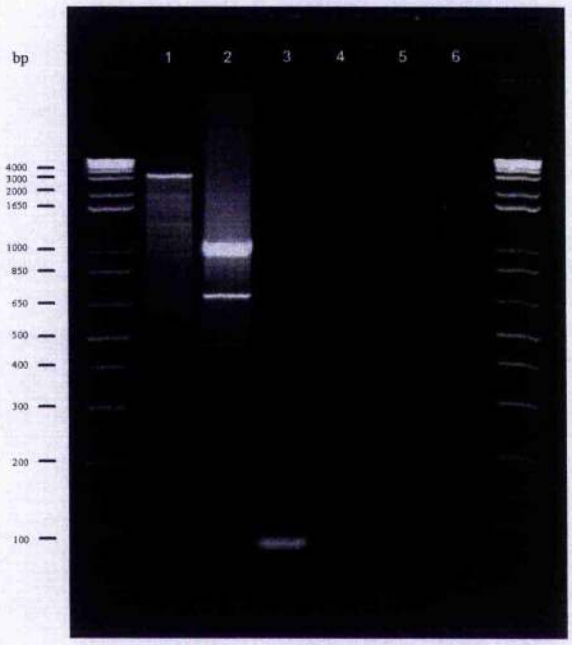


Figure 5.5. *5'RACE PCR*

Analysis of 1/10 (v/v) of each PCR in a 1.2% (w/v) agarose gel stained with Gelstar nucleic acid. The molecular weight marker was 0.1µg 1kb+ DNA ladder (Invitrogen). The first three reactions were performed on Clontech rat brain cDNA template using the following primers: 1) Clontech Adapter Primer (AP) and gene-specific reverse primer, 2) Adapter Primer (AP) and a *GAPDH* 5' RACE control primer (positive control), 3) EST-specific primer for RACE and the forward primer used in qRT-PCR (positive control). The last three reactions were performed without any template using the following primers: 4) the Adaptor Primer (AP) alone (negative control), 5) the EST-specific primer for RACE alone (negative control) and 6) same primers as 1).

The length of the obtained 3.4kb RACE amplicon was analysed in the context of our bioinformatics analyses.

Indeed, at the time when these analyses were first performed, rat *RGD1308367* gene was mapped to Chr.3, 39719751 to 39724482 and was predicted to have 3 exons spanning from 39719751 to 39720268, 39723562 to 39723654 and 39724239 to 39724482 respectively (which gives a predicted cDNA of 852bp). The EST AI072720 sequence aligned with rat genomic sequence on Chr.3, 39717303 to 39717688, *i.e.* 2063bp downstream from RGD1308367 last predicted exon. Therefore, amplification of a ~3kb (862bp + 2063bp = 2915bp) transcript from the EST sequence was consistent with AI072720 being part of exon 3 and most likely of a long 3'UTR sequence. If EST AI072720 was an alternative exon or splice variant of RGD1308367, the 5' RACE PCR would have given a much shorter amplicon since intervening intronic sequence would not have been amplified by the RACE PCR.

Reamplification of the RACE product using nested primers (figure 5.6) gave a ~3kb-long product (data not shown), consistent with the expected predicted size.

Sequencing of this nested EST AI072720/RGD1308367 PCR product was not performed due to the very low yield of the 3kb amplicon. However, successful amplification of a product of the expected length from the RACE product template using gene-specific nested primers provided further evidence that the EST was part of *RGD1308367*.

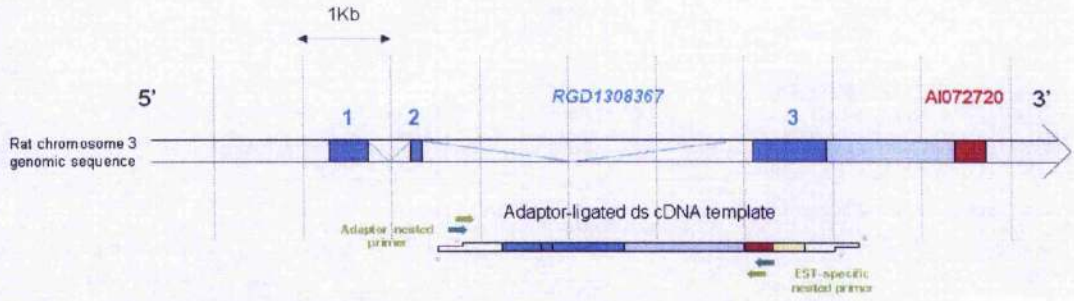


Figure 5.6. *Position of primers used to reamplify the RACE product*

In order to reamplify the 5' RACE PCR product, a reverse EST-specific nested primer was designed and used together with forward adapter nested primer (Clontech). The 3 exons of *RGD1308367* are numbered as 1, 2 and 3. Within the adaptor-ligated ds cDNA template, marathon cDNA adapters are represented in white while the primer used for synthesising the cDNA is shown in yellow.

Since we had not been able to get the RACE product sequenced, a series of PCRs were performed to confirm that EST AI072720 was part of *RGD1308367* 3'UTR sequence. Rat brain cDNA was used as a template for amplifying 1) the full-length *RGD1308367* transcript with its 3'UTR, 2) concatenated *RGD1308367* second and third exons with 3'UTR and 3) *RGD1308367* third exon with 3'UTR only. Three different combinations of primers were used to amplify these regions (Figure 5.7).

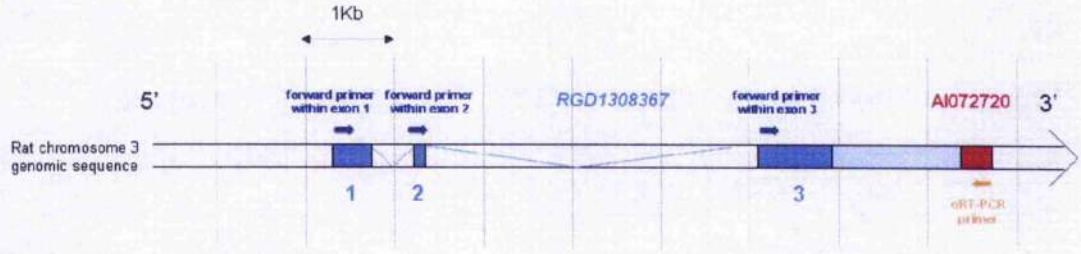


Figure 5.7. *Position of primers used for the different PCRs on rat brain cDNA*

To confirm that EST AI072750 was part of *RGD1308367*, the reverse primer used for qRT-PCR on EST AI072720 was used in different PCRs on rat brain cDNA in combination with forward primers respectively located within each of the 3 exons of *RGD1308367*. The position of each of these primers is shown on this figure. According to the hypothesis that EST AI072720 was part of *RGD1308367* 3'UTR, the expected sizes of amplicons were 3098bp, 2894bp and 2820bp for PCRs performed using the qRT-PCR reverse primer and forward primers located within *RGD1308367* first, second and third exon respectively.

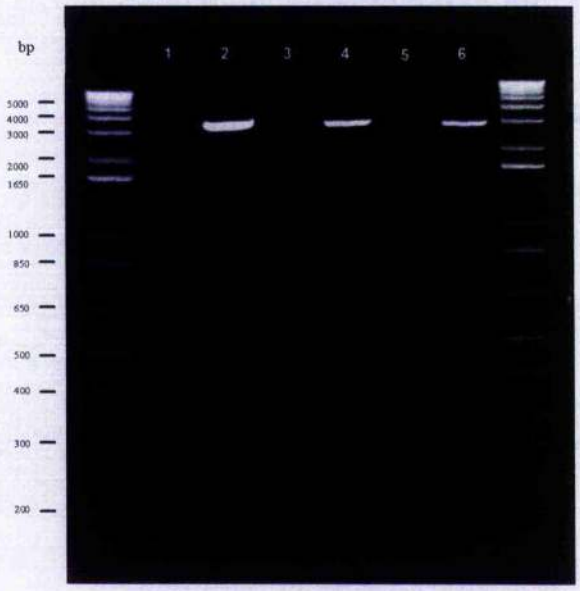


Figure 5.8. *PCRs on rat brain cDNA*

Analysis of 4% (v/v) of each PCR product in a 1.2% (w/v) agarose gel stained with Gelstar nucleic acid. The molecular weight marker was 0.1µg 1kb+ DNA ladder (Invitrogen). Lanes 1, 3 and 5 show PCR products amplified from rat brain cDNA using the qRT-PCR reverse primer and a forward primer within the first, second or third exon of *RGD1308367*, respectively. Bands of approximately 3.1kb (lane 2), 2.9kb (lane 4) and 2.8kb (lane 6) were obtained, consistent with the predicted sizes. Lanes 2, 4 and 6 represent the same reactions, respectively, but performed without any template (negative controls).

PCR with qRT-PCR reverse primer and forward primer within the first exon of *RGD1308367* was repeated in order to sequence the 3.1kb full-length *RGD1308367* transcript with its 3'UTR (Figure 5.9).

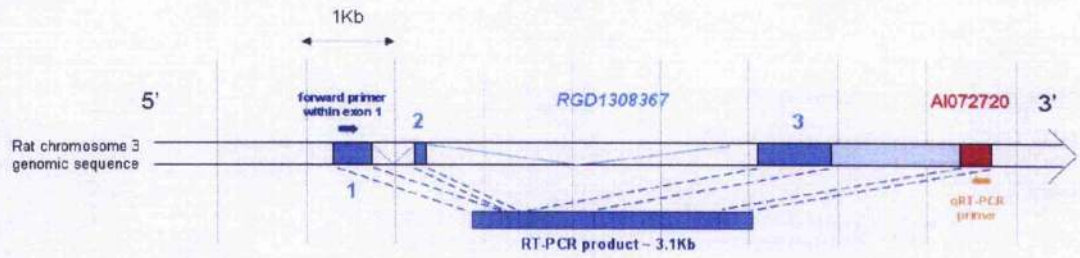


Figure 5.9. *PCR product amplified with qRT-PCR reverse primer and forward primer within the first exon of RGD1308367*

Sequencing of this PCR product confirmed that EST AI272720 was part of *RGD1308367* 3'UTR. The sequence 5' of the EST exactly corresponded to the genomic sequence between exon 3 of *RGD1308367* and the EST (shaded blue in Figure 5.9) and continued into exon 3, exon 2 and exon 1 with no intronic DNA included.

5.4- Validation of *RGD1308367* gene: qRT-PCR (rat/human), *in situ* hybridisation

Confirmation of EST AI072720 as part of a rat transcript *RGD1308367* led us to hypothesize that *RGD1308367* might also be differentially regulated in the prefrontal cortex after chronic PCP treatment. This was investigated by qRT-PCR and semi-quantitative *in situ* hybridisation.

A rat qRT-PCR assay was designed within *RGD1308367* coding sequence to investigate *RGD1308367* expression in the rat prefrontal cortex after chronic PCP treatment (section 2.4.5). The assay was performed on an independent set of rat prefrontal cortex samples (not previously used for the microarray study). qRT-PCR showed significant upregulation of *RGD1308367* after chronic PCP treatment ($p=0.031$, +45.8%) which was consistent with the microarray data and with previous results showing differential expression of EST AI072720 in the rat prefrontal cortex after chronic PCP treatment (section 3.4.3).
This result was therefore consistent with the EST AI072720 being part of *RGD1308367* transcript and that its differential expression after PCP treatment (as found in the microarray) could actually be reflecting *RGD1308367* expression.

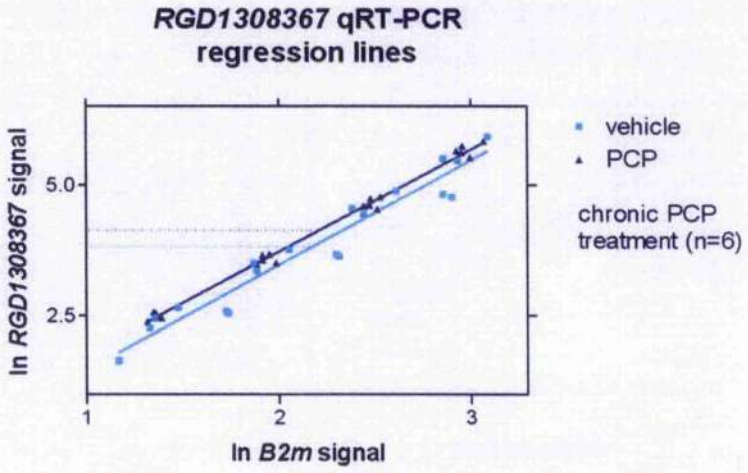


Figure 5.10. *Regression lines for rat RGD1308367 qRT-PCR*

qRT-PCR was performed to evaluate *RGD1308367* expression changes in the rat prefrontal cortex after chronic PCP treatment by comparison with a vehicle (saline solution) (n=6). Y-axis is the logarithm of the fluorescence intensity representing the expression of *RGD1308367* and X-axis is the logarithm of the fluorescence intensity for *beta-2-microglobulin (B2m)*, the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which revealed 45.8% increase in *RGD1308367* expression after chronic PCP treatment (p=0.031). Dotted lines show an example of increased *RGD1308367* signal for a particular *B2m* signal.

A human qRT-PCR assay from the coding region was purchased for detecting the expression of *KIAA1189* (*RGD1308367* human orthologue) in human post-mortem dorso-lateral prefrontal cortex (DLPFC) from schizophrenic patients. Differential expression was confirmed in a subset of the samples (n=5) corresponding to two populations of schizophrenic patients, namely the ones collected by Harvard and Brain-Net brain bank organisations (Figure 5.11). *KIAA1189* expression was found to be significantly upregulated in schizophrenic patients compared to controls (p=0.001, +14%) which was consistent with *RGD1308367* upregulation in the rat prefrontal cortex after chronic PCP treatment.

Interestingly, *KIAA1189* differential expression in schizophrenic patients was therefore confirmed using this assay designed within *KIAA1189* coding sequence whereas it had not been shown using a different qRT-PCR assay detecting *KIAA1189* 3'UTR (section 3.4.3).

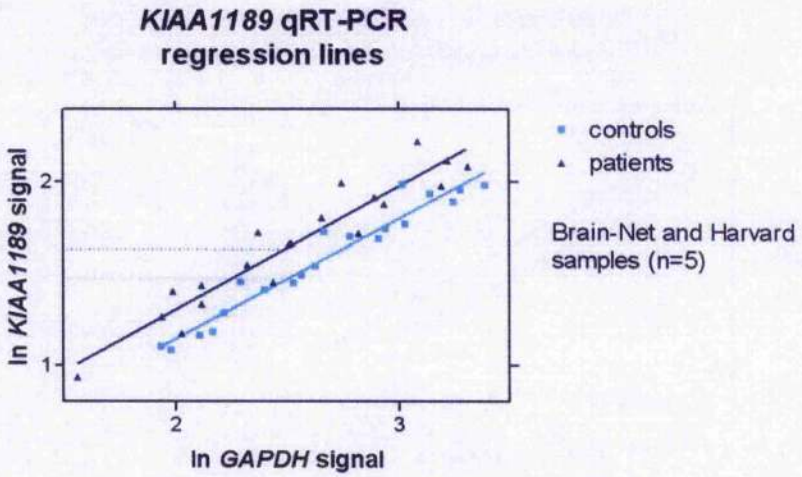


Figure 5.11. *Regression lines for human KIAA1189 qRT-PCR*

qRT-PCR was performed to evaluate *KIAA1189* expression changes in human post-mortem dorso-lateral prefrontal cortex from schizophrenic patients compared to controls (n=9 in total, n=5 when considering schizophrenic patients from the Brain-Net and Harvard collections only). Y-axis is the logarithm of the fluorescence intensity representing the expression of *KIAA1189* and X-axis is the logarithm of the fluorescence intensity for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the internal control gene that was used for normalisation. For each gene, the graph plots four points taken from the linear amplification phase of the PCR. The data were analysed by ANCOVA, which revealed 14% increase in *KIAA1189* expression in schizophrenic patients from the Brain-Net and Harvard collections (n=5, p=0.001). Dotted lines show an example of increased *KIAA1189* signal for a particular *GAPDH* signal.

In situ hybridisation was performed on *RGD1308367* using probes designed within the coding sequence of the gene. Two sets of films were developed in order to measure the higher white matter expression and lower prefrontal cortex expression after different exposure times.

Figure 5.12 shows the regional distribution of *RGD1308367* mRNA in the rat brain. This gene was found to be highly expressed within all major white matter tracts. Within cortical and subcortical areas, it was expressed at a much lower level and had a homogeneous distribution. Expression was observed within all cortical areas, the striatum, thalamus, hypothalamus and hippocampus, with the prefrontal cortex appearing to display the lowest level.

This discrete expression pattern looked consistently identical to the expression pattern of EST AI072720 (section 3.4.3).

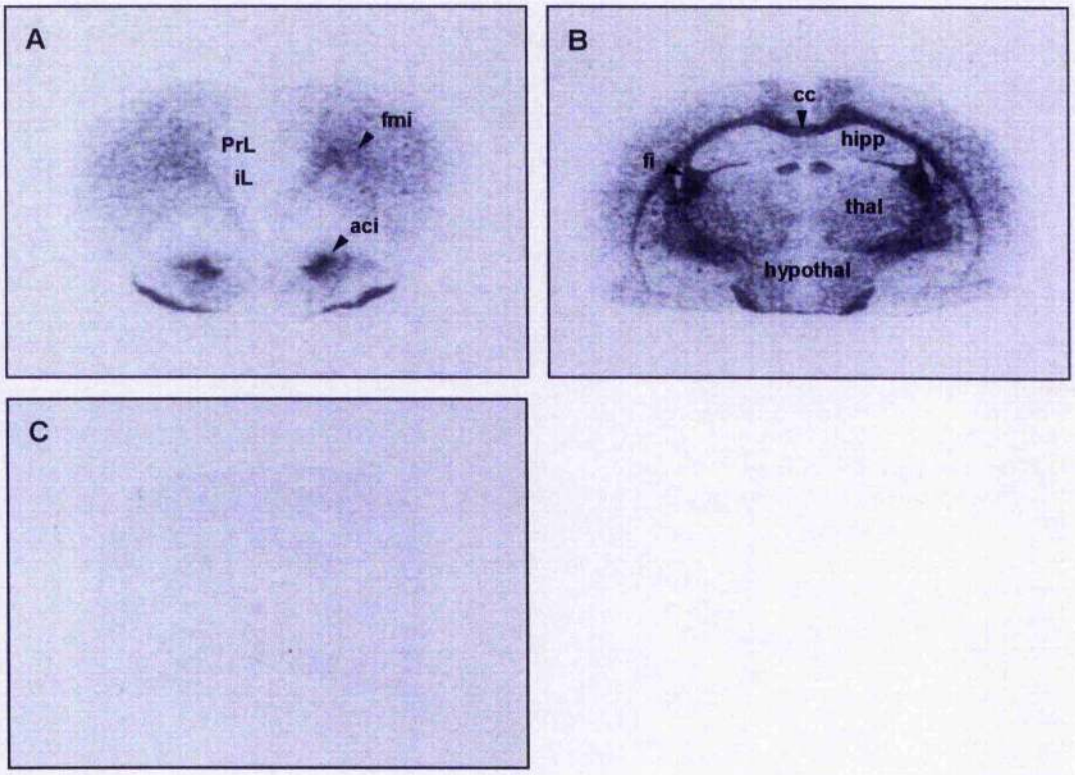


Figure 5.12. *Representative sections illustrating the regional distribution of RGD1308367 mRNA in selected regions of the rat brain (panels A and B) and control section (panel C)*

Coronal sections used for *RGD1308367 in situ* hybridisation were obtained from the following bregma levels according to Paxinos (1998): A) 3.20mm (prefrontal cortex), B) -2.30mm (midline thalamus) (Paxinos 1998). Sections were hybridised with ^{35}S-labelled oligonucleotide probes specific for *RGD1308367* (in presence of an excess of unlabelled probe in panel C), exposed to film for two different periods of time (one for reading white matter and one for detecting prefrontal cortex expression) and digitally scanned. Black areas mark the presence of mRNA.
PrL: prelimbic cortex; iL: infralimbic cortex; fmi: forceps minor corpus callosum; aci: intrabulbar part of the anterior commisure: cc: corpus callosum; fi: fimbria of hippocampus; hipp: hippocampus; thal: thalamus; hypothal: hypothalamus.
The uniform, non-specific signal shown in panel C) provides confirmation of the specificity of the probes used for *RGD1308367 in situ* hybridisation studies.

28 regions of the rat brain were analysed for differential expression of *RGD1308367* expression : prelimbic cortex (PrL), infralimbic cortex (iL), ventral orbital cortex (vO), lateral orbital cortex (lO), primary and secondary motor cortices (M1 and M2), anterior cingulate cortex (acg), granular (rsg) and agranular (rsa) retrosplenial cortices, dentate gyrus granule cell layer (DGgcl), pyramidal cell layers of the CA1 (CA1pcl), CA2 (CA2pcl) and CA3 (CA3pcl) region of the hippocampus, primary auditory cortex (Au1), lateral entorhinal cortex (Lent), corpus callosum (cc), genu of corpus callosum (gcc), forceps minor (fmi) and forceps major (fmj) corpus callosum, anterior commisure (ac), intrabulbar part of the anterior commisure (aci), external (ec) and internal (ic) capsules, cingulum (cg), stria medullaris of the thalamus (sm), mammillothalamic tract (mt), fornix (f) and fimbria of hippocampus (fi).

In two white matter regions, the fornix (f) and the forceps minor of the corpus callosum (fmi, Figure 5.12A), chronic PCP administration significantly decreased *RGD1308367* expression ($p=0.006$ for the forceps minor corpus callous, $p=0.008$ for the fornix), suggesting that treatment with PCP can indeed modulate the expression of *RGD1308367* mRNA.

Although it was not significant, there was a trend for increased expression of *RGD1308367* in the anterior cingulate cortex ($p=0.067$) and a trend for a decreased expression in the primary motor cortex ($p=0.065$).

These results differed from those of EST AI072720 *in situ* hybridisation (section 3.4.3), where no significant change was found in the expression levels of EST AI072720 in any region.

In conclusion, these results show the confirmation of *RGD1308367* differential upregulation in the rat prefrontal cortex after chronic PCP treatment (qRT-PCR results) and provide evidence of a regulation of its expression in white matter regions as well (*in situ* hybridisation data). Interestingly, qRT-PCR analysis of *KIAA1189* (*RGD1308367* human orthologue) expression in the dorso-lateral prefrontal cortex (DLPFC) of schizophrenic patients provided the first confirmation of the concomitant differential expression of a rat gene and of its human orthologue in the rat prefrontal cortex after chronic PCP treatment and in the analogous human brain region (DLPFC) of schizophrenic patients.

5.5- *In silico* characterisation of *KIAA1189* gene and KIAA1189 protein

As a first step towards the characterisation of *RGD1308367/KIAA1189*, bioinformatics tools were used to do more data mining on both rat and human genes and proteins. GeneCards and Harvester were used to retrieve all available information about the genes, their expression and protein function.

Human *KIAA1189* gene (name approved by HUGO Gene Nomenclature Committee in February 2004) was found to map to chromosome 2q24.1. Interestingly, chromosome 2q, from 2q11 to 2q37, has been linked with schizophrenia in five independent genome scans (Levinson *et al.* 1998; Williams *et al.* 1999; Mowry *et al.* 2000; DeLisi *et al.* 2002a; DeLisi *et al.* 2002b; Wijsman *et al.* 2003) and it was the only region of the genome that reached genome-wide significance in a meta-analysis (Lewis *et al.* 2003). Moreover, the 2q locus has recently been associated with visual working memory in a linkage study looking at cognitive trait components of schizophrenia (Paunio *et al.* 2004).

Figure 5.13 represents *KIAA1189* gene expression in human tissues according to GeneNote (twelve normal human tissues hybridised to Affymetrix GeneChips HG-U95A-E) and table 5.1 shows the normalised expression distribution of *KIAA1189* for tissue type according to UniGene (Source @ Stanford University at Harvester).

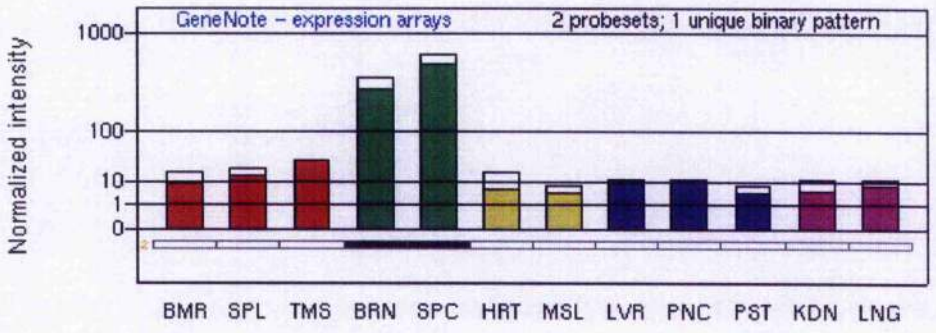


Figure 5.13. *KIAA1189 expression in human tissues*

(BMR: bone marrow, SPL: spleen, TMS: thymus, BRN : brain, SPC: spinal cord, HRT: heart, MSL: skeletal muscle, LVR: liver, PNC: pancreas, PST: prostate, KDN: kidney, LNG: lung)

Tissue	Normalized Expression (%)	Cluster Clones : Tissue clones
Brain:	70.33	41:319574
Lymph_Node:	19.26	4:113869
mixed:	5.23	3:314320
other:	5.17	4:423795

Table 5.1. *KIAA1189 normalised expression in different tissues (UniGene)*

Both sources gave consistent information suggesting that *KIAA1189* is predominantly expressed in the brain and in the spinal cord in comparison with other tissues of the body.

At many websites including Harvester (Smart analysis) and Ensembl (InterPro domain), both *RGD1308367* and *KIAA1189* putative proteins were predicted to have a carboxy-terminal Ezrin-Radixin-Moesin (ERM) domain. This domain, typically present in ERM proteins in conjunction with an N-terminal membrane-binding domain, is known to bind actin which suggested that *KIAA1189* may have a role in cytoskeletal architecture.

Finally, TrEMBL was found to predict two proteins (corresponding to two alternative transcripts) for KIAA1189: a 284 amino acid (32783kDa) protein, Q8TAM6 (Refseq NP_001009959.1) and a 300 amino acid (34584 Da) Q9ULN1 protein (Refseq NP_065762.1). Both proteins were predicted to have a moesin domain (Interpro IPR008954).

These preliminary *in silico* analyses of *KIAA1189* therefore provided evidence that this gene is very highly expressed in the brain and that its locus (2q) has been strongly linked with schizophrenia. Moreover, a putative role of KIAA1189 in cytoskeletal architecture (as suggested from its C-terminal ERM domain) may be consistent with cell morphology abnormalities observed in schizophrenia.

Therefore, *RGD1308367/KIAA1189* appeared a very intriguing novel gene for which to investigate in relation with schizophrenia.

5.6- *KIAA1189* cDNA cloning and overexpression

Molecular biology studies *in vitro* including cDNA cloning, expression and functional analyses were undertaken in order to functionally characterise *RGD1308367/KIAA1189* in relation with schizophrenia.

5.6.1- Generation of pcDNA3.1-FLAG®-KIAA1189 expression construct

In the absence of commercially available RGD1308367 or KIAA1189 antibodies, a mammalian expression construct containing a FLAG® tag, pcDNA3.1-FLAG®, was used as a vector to sub-clone the full-length human *KIAA1189* transcript, so that FLAG®-KIAA1189 could be detected by N-terminal FLAG® immunoreactivity.

A full-length human *KIAA1189* cDNA clone was purchased as a plasmid (pBluescriptR) in *E. Coli* and its identity was verified by sequencing as being identical to Genbank mRNA sequence BC026345 *i.e. KIAA1189* longest mRNA sequence (data not shown). Primers containing 5'- and 3'-end restriction enzyme recognition sites (for *KpnI* and *XhoI* respectively) were designed for allowing in frame cloning of *KIAA1189* cDNA into the pcDNA3.1-FLAG® expression vector and were used to amplify *KIAA1189* from its original pBluescriptR vector.

A PCR product of 0.9kb (the expected length) and pcDNA3.1-FLAG® vector were digested with *KpnI* and *XhoI* and ligated (section 2.4.9).

TOP10 bacterial cells were transformed with pcDNA3.1-FLAG®-KIAA1189 ligation reactions (section 2.5.1) and the cells were checked for the presence of the expression construct. Plasmid DNA was extracted from twenty-four colonies (section 2.4.1.1) and digested with *SpeI* to discriminate the plasmids containing *KIAA1189* insert from the ones without. Thirteen pcDNA3.1-FLAG® plasmids were shown to contain *KIAA1189* the cDNA insert. The construct was sequenced across the vector/insert junction which confirmed that the correct reading frame had been maintained.

5.6.2- Expression of pcDNA3.1-FLAG®-KIAA1189

In order to investigate the expression of KIAA1189 *in vitro*, rat C6 glioma cells were transfected with pcDNA3.1-FLAG®-KIAA1189 (section 2.5.4.1). Cell lysate was extracted after 24h and 48h (section 2.5.6) and proteins were quantified using the Bradford dye-binding assay (section 2.5.8).

Western blotting was used to assay FLAG®-KIAA1189 overexpression in transfected cells. Samples containing 1μg of protein were subjected to electrophoresis in a 10% (w/v) SDS-PA gel and transferred by blotting to PVDF membranes (section 2.5.9.2). The blots were analysed with 1/5000 dilution of an HRP-conjugated FLAG® antibody. A FLAG®-tagged protein of approximately 32kDa was immunodetected (Figure 5.14, lanes 2 and 3) consistent with KIAA1189's expected molecular weight (~32kDa), suggesting that *KIAA1189* had been translated as a FLAG® fusion protein in these cells. Expression of this FLAG®-KIAA1189 protein was higher at 24h (lane 2) than at 48h (lane 3) after transfection, making 24h a better time point for looking at KIAA1189 overexpression using C6 cells.

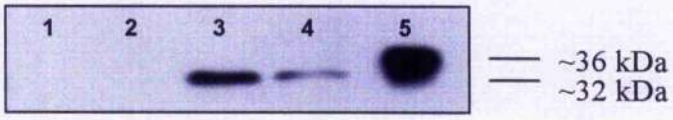


Figure 5.14. *Western blotting showing FLAG®-KIAA1189 overexpression in rat C6 glioma cells*

Lanes 1 and 2 are negative controls consisting of cell lysate from untransfected C6 cells (lane 1) and cells transfected with the empty pcDNA3.1-FLAG® expression vector (lane 2). Lanes 3 and 4 contain lysate from FLAG®-KIAA1189-transfected cells, lysate having been extracted at 24h and 48h (respectively). Lane 5 is a positive control consisting of cell lysate from C6 cells transfected with a control pcDNA3.1-FLAG®-NCK1 construct. The FLAG® antibody immunodetected a FLAG® immunoreactive FLAG®-NCK1 protein of ~36kDa (expected molecular weight).

5.7- Immunocytochemical analyses of KIAA1189 subcellular localisation in C6 cells

FLAG® immunofluorescence on pcDNA3.1-FLAG®-KIAA1189 transfected cells was used to investigate the cellular localisation of KIAA1189 protein and to investigate its potential colocalisation with actin, predicted from the presence of a carboxy-terminal ezrin-radixin-moesin (ERM) domain.

Rat C6 glioma cells were transfected with pcDNA3.1-FLAG®-KIAA1189 expression construct (section 2.5.4.1) and processed for immunofluorescence (section 2.5.12). After fixation, permeabilisation and blocking of non-specific adsorption of the antibodies, the cells were incubated with 1/2000 dilution of mouse monoclonal anti-FLAG® M2 antibody followed by a 1/400 dilution of polyclonal anti-mouse IgGs conjugated with Alexa Fluor® 596 (both incubations were 1 hour at room temperature). Fluorescently labelled (Alexa Fluor® 488-conjugated) phalloidin, a toxin from the deadly *Amanita phalloides* mushroom which specifically binds filamenteous actin, was used for labelling F-actin and 4',6-diamidino-2-phenylindole (DAPI) was utilised for staining cell nuclei (section 2.5.12).

Figure 5.15 shows confocal microscopy images of C6 glioma cells transfected with pcDNA3.1-FLAG®-KIAA1189 and processed for FLAG® immunofluorescence and phalloidin staining.

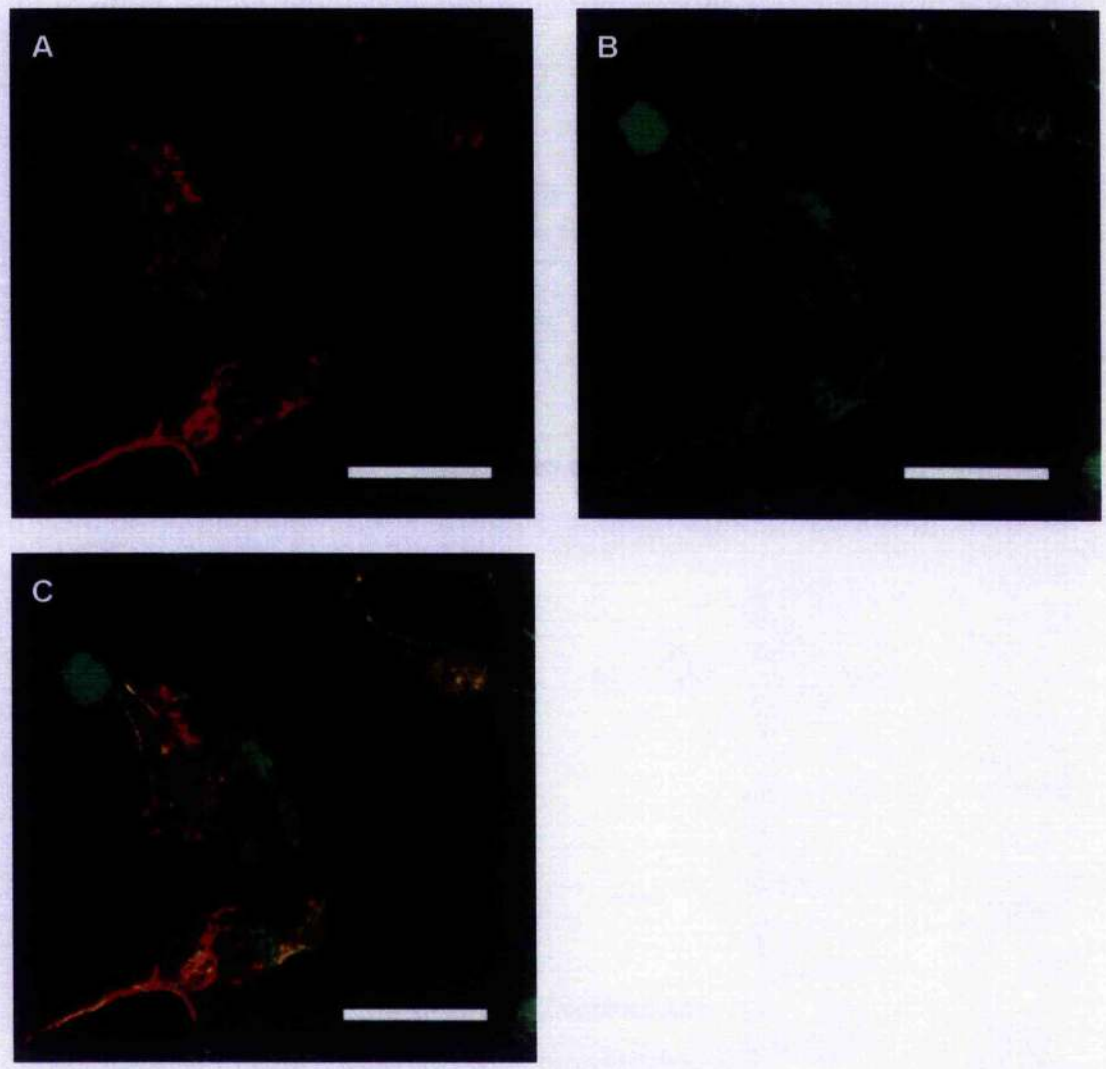


Figure 5.15. *Confocal immunofluorescence images of rat C6 glioma cells expressing the FLAG®-KIAA1189 fusion protein*

Rat C6 glioma cells transfected with FLAG®-KIAA1189 were processed for FLAG® immunofluorescence and phalloidin staining. Panel A shows FLAG® labelling in red as immunodetected by the FLAG® M2 antibody conjugated with Alexa Fluor® 596. Panel B represents F-actin staining in green as detected by Alexa Fluor® 488-conjugated phalloidin. Panel C is the merged image of panels A and B, where the yellow-orange represents merging of the red FLAG® immunofluorescence and the green phalloidin staining. Scale bar represents 50μm.

FLAG®-KIAA1189 was found to be expressed either in the plasma membrane or very close to it and its expression was higher in all neuronal processes than around the cell body (Figure 5.15).

Colocalisation of FLAG®-KIAA1189 with actin was not confirmed throughout the cells but was confirmed in the plasma membrane and in some neuronal-like processes.

5.8- Identification of potential KIAA1189 interacting proteins by immunoprecipitation

Immunoprecipitation was used in order to assess possible binding of KIAA1189 with actin and identify other potential KIAA1189-interacting proteins, thereby deciphering the putative function of KIAA1189 protein through *in vitro* studies.
In the absence of any commercially-available antibody, highly specific FLAG® immunoreactivity was used to precipitate FLAG®-KIAA1189 fusion protein together with the proteins that may complex with it. This experiment was carried out using rat C6 glioma cells transfected with pcDNA3.1-FLAG®-KIAA1189 (section 2.5.4.1) and the cells were lysed 24h after transfection (section 2.5.6). Purification of a control fusion FLAG®-Bacterial Alkaline Phosphatase (FLAG®-BAP) protein (Sigma-Aldrich) was performed alongside experimental samples.
Isolated and purified proteins were subjected to electrophoresis in a 10% (w/v) SDS-PA gel and transferred by blotting to PVDF membranes (section 2.5.9). The blots were first analysed with 1/5000 dilution of an HRP-conjugated FLAG® antibody.
As expected, FLAG®-tagged proteins of ~32kDa and ~50kDa were detected corresponding to the FLAG®-KIAA1189 and FLAG®-BAP fusion proteins respectively, which validated the immunoprecipitation protocol.

The same samples were then again subjected to electrophoresis, transferred to PVDF membrane and analysed with a 1/1000 dilution of a rabbit anti-(pan)-actin antibody followed by 1/2000 dilution of an anti-rabbit HRP-linked antibody. A ~42kDa protein corresponding to actin was detected in the positive control sample consisting of a non-immunoprecipitated cell lysate. However actin was not detected in the immunoprecipitated sample from pcDNA3.1-FLAG®-KIAA1189 transfected cells suggesting it was not part of the FLAG®-immunoprecipitated complex.
The experiment was repeated several times and gave the same results.

Because of this failure to detect actin within the FLAG®-immunoprecipitated complex (from C6 cells transfected with pcDNA3.1-FLAG®-KIAA1189), Coomassie blue staining was performed on SDS-PA electrophoresed samples to detect and visualise all immunoprecipitated proteins. Very surprisingly, only a single band was visible after several hours of staining, whose molecular weight was consistent with the ~32kDa molecular weight of the FLAG®-KIAA1189 fusion protein, suggesting that no other proteins had been immunoprecipitated.
To confirm this hypothesis, both the band excised from the SDS-PA gel and the whole FLAG®-immunoprecipitated sample were analysed by mass spectrometry. Apart from a very common keratin contamination (due to human manipulation), the only peptides that were identified by this method were derived from KIAA1189 protein and mouse monoclonal IgGs (resulting from insufficient purification of the FLAG® antibody during the immunoprecipitation protocol).

These preliminary experiments did not confirm interaction of KIAA1189 with actin and did not identify any other potential interacting partners of this protein.

5.9- Immunocytochemical analyses of KIAA1189 subcellular localisation in PC12 cells

Failure to confidently confirm KIAA1189 interaction with actin or uncover any of KIAA1189 potential functions using C6 glioma cells led to the use of a more neuronal-like cell type, rat PC12 cells, for similar overexpression studies.

Figure 5.16 shows confocal microscopy images of differentiated PC12 cells transfected with pcDNA3.1-FLAG®-KIAA1189 and processed for FLAG® immunofluorescence and phalloidin staining.

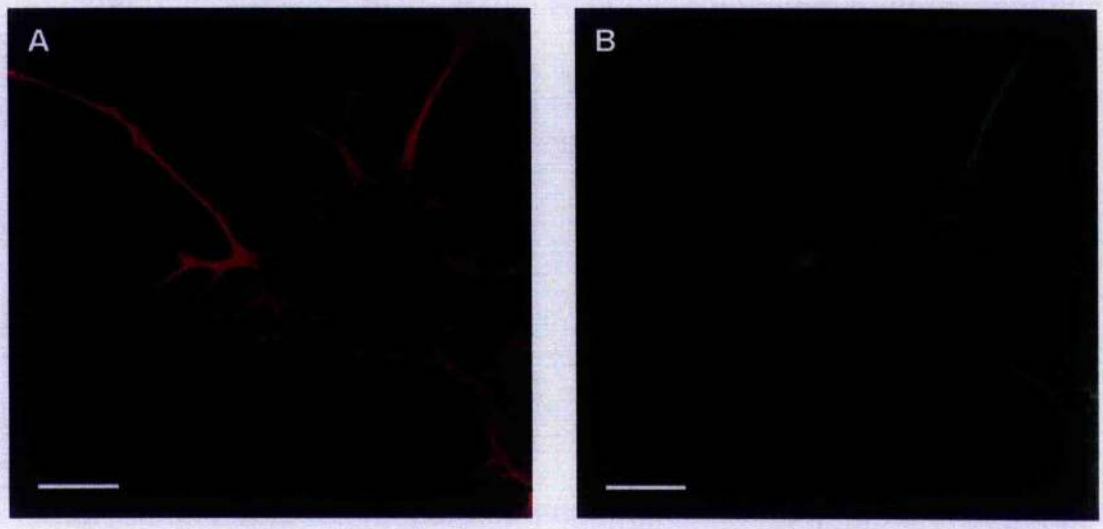


Figure 5.16. *Confocal immunoflorescence images of rat differentiated PC12 cells expressing the FLAG®-KIAA1189 fusion protein*

Rat differentiated PC12 cells transfected with FLAG®-KIAA1189 were processed for FLAG® immunofluorescence and phalloidin staining. Panel A shows FLAG® labelling in red as immunodetected by the FLAG® M2 antibody conjugated with Alexa Fluor® 596. Panel B represents F-actin staining in green as detected by Alexa Fluor® 488-conjugated phalloidin. Scale bar represents 20μm.

In differentiated PC12 cells, FLAG®-KIAA1189 was found to be predominantly expressed in neurites (with a very high expression in some but not all tips of potentially extending neurites) and in the plasma membrane and exhibited very weak expression within the cell body. By comparison, the expression pattern of actin was more homogeneous, with lower expression within the processes but higher expression all over the cell body, which is consistent with the essential role of actin in cytoskeletal architecture.

As shown in figure 5.17 (merged FLAG® and phalloidin fluorescence), FLAG®-KIAA1189 did colocalise with actin in some but not all neuronal-like processes and in the plasma membrane which was consistent with the observations made using C6 cells. FLAG®-KIAA1189 also colocalised with actin at the end of some neurite tips (figure 5.17).

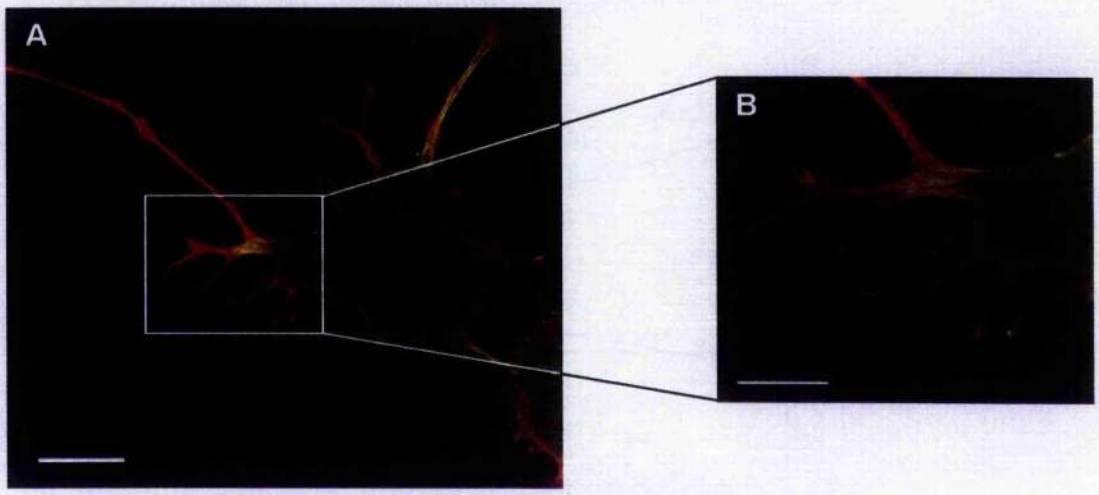


Figure 5.17. *Confocal immunofluorescence images of rat differentiated PC12 cells expressing the FLAG®-KIAA1189 fusion protein*

Rat differentiated PC12 cells transfected with FLAG®-KIAA1189 were processed for FLAG® immunofluorescence and phalloidin staining. These images represent merged images of the red (Alexa Fluor® 596) FLAG® immunolabelling and the green (Alexa Fluor® 488) F-actin staining, the areas where both stainings coincide showing up in yellow-orange. Panel B (scale bar: 10μm) shows a detail of the whole differentiated PC12 cell represented in panel A (scale bar: 20μm).

All together, this suggested that KIAA11889 may play a role in relation to peripheral actin at the membrane and in neurites which may be consistent with a structural role at the membrane and/or a role in actin-based cell migration events such as neurite extension.

5.10- Neurite outgrowth assay

A potential role for KIAA1189 in neurite outgrowth was investigated *in vitro* using PC12 cells overexpressing KIAA1189 and undergoing NGF-induced differentiation. This cell line of neuroepithelial origin has been widely used and characterised as an *in vitro* model of neurite extension (Kiryushko *et al.* 2004).

A neurite outgrowth quantification kit was used for semi-quantitatively evaluating the effect of KIAA1189 overexpression on neurite extension (section 2.5.13) while cotransfection of pcDNA3.1-FLAG®-KIAA1189 and GFP was performed to examine the effect of KIAA1189 overexpression on cell morphology (including

neurite length). In both cases, neurite outgrowth in KIAA1189-overexpressing cells was investigated in comparison with cells that had been transfected with the empty pcDNA3.1-FLAG® expression vector.

Quantification of neurite outgrowth was performed at different times (1, 2, 3, 5 and 7 days) after the initiation of PC12 differentiation in order to investigate the effects of KIAA1189 overexpression on the time course of neurite outgrowth. The experiment was repeated and gave a similar pattern of time course for pcDNA3.1-FLAG®-KIAA1189 and pcDNA3.1-FLAG®-transfected cells (figure 5.18).

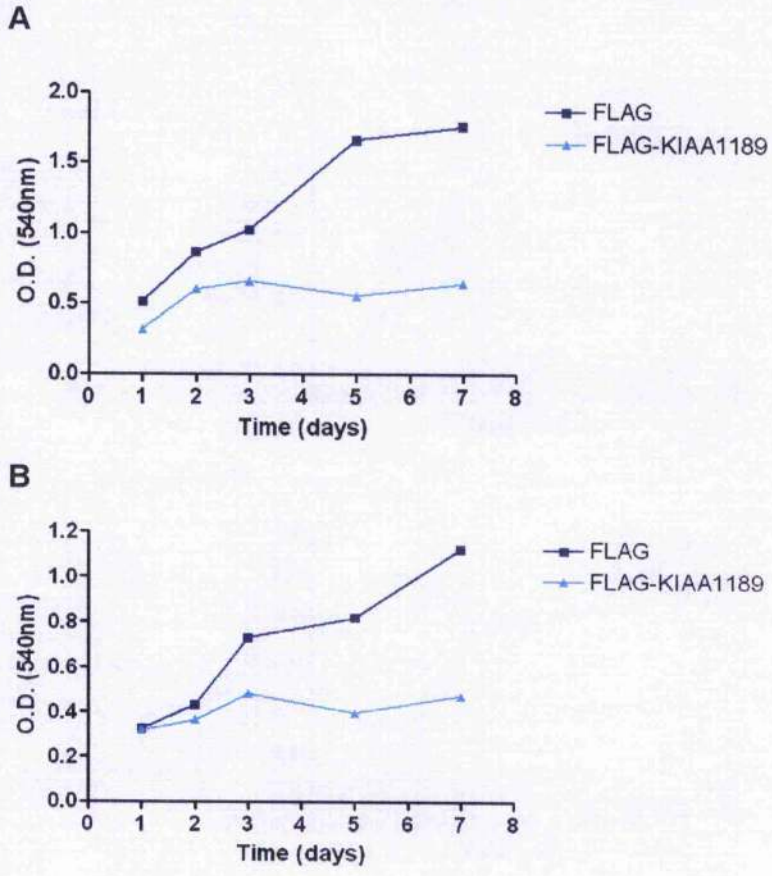


Figure 5.18. *Time course of neurite outgrowth of differentiating PC12 cells*

To examine the effects of KIAA1189 on neurite outgrowth, PC12 cells were plated onto a microporous membrane, transfected with FLAG®-KIAA1189 or the empty pcDNA3.1-FLAG® expression vector and PC12 differentiation was initiated by the addition of NGF to the cell culture medium. Neurites were extracted at 1, 2, 3, 5 and 7 days after transfection and quantitatively analysed by optical density (O.D.) at 540nm.

The graphs show time courses of PC12 cells neurite outgrowth quantified as such, the experiment was performed twice (panels A and B).

Over time, pcDNA3.1-FLAG®-KIAA1189-transfected cells were found to extend less and/or fewer neurites than pcDNA3.1-FLAG®-transfected cells suggesting that KIAA1189 overexpression inhibits or prevents neurite outgrowth.

In order to confirm this result, PC12 cells were cotransfected with pcDNA3.1-FLAG®-KIAA1189 or pcDNA3.1-FLAG® together with GFP and analysed for cell morphology changes using GFP immunofluorescence. FLAG®-KIAA1189 overexpression did not produce any morphological abnormalities but although no statistical analysis was performed, KIAA1189-overexpressing cells appeared to exhibit shorter neurites than FLAG®-overexpressing cells (figure 5.19), which is consistent with previous results using the neurite outgrowth kit quantification.

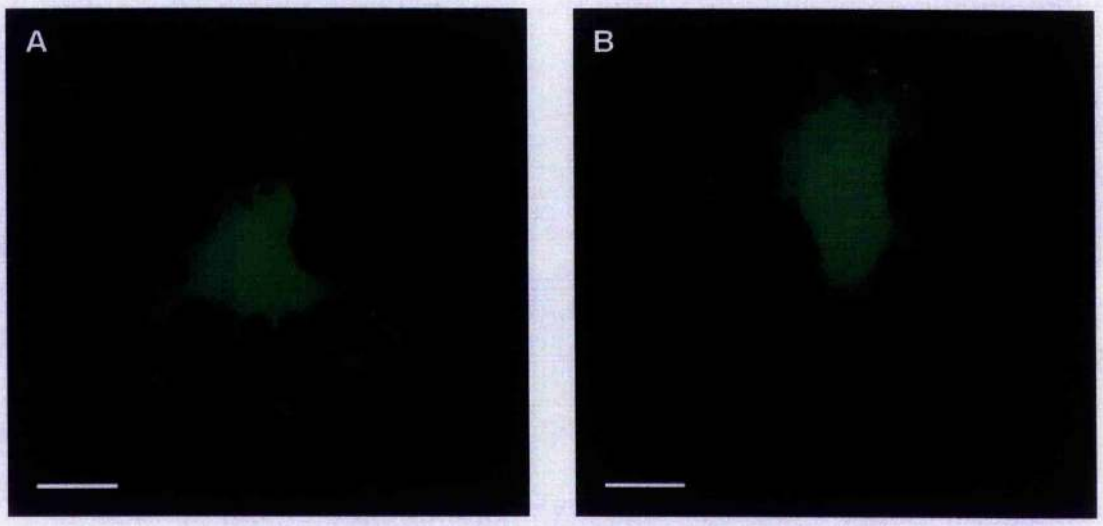


Figure 5.19. *Immunofluorescence images of rat differentiated PC12 cells transfected with the empty FLAG® expression vector (A) or FLAG®-KIAA1189 fusion protein (B) together with GFP*

GFP epifluorescence (green colour on these images) was used to investigate the cell morphology of rat differentiated PC12 cells cotransfected with the empty pcDNA3.1-FLAG® expression vector (A) or FLAG®-KIAA1189 fusion protein (B) together with GFP. Scale bar is 10μm

These experiments using differentiating PC12 cells provided evidence of a role of KIAA1189 in neurite outgrowth or inhibition of neurite outgrowth. Such a function is consistent with high KIAA1189 expression within neurite tips in PC12 cells (section 5.9) and may be related to a potential interaction between KIAA1189 and F-actin.

5.11- KIAA1189 SNP analysis

SNP genotyping was performed in order to investigate any genetic association of KIAA1189 with schizophrenia.

Average allele frequencies were used for choosing the more informative SNPs to examine, and three allelic discrimination assays were purchased that are listed in the dbEST database (NCBI) under the IDs rs1867846 (Applied Biosystems ID c_11635778_10), rs13028288 (Applied Biosystems ID c_3254608_30) and rs12613898 (Applied Biosystems ID c_31158365_10).

SNP genotyping was performed (section 2.4.8) on a set of 600 samples from schizophrenic patients and controls (300 patients, 300 controls) obtained from Professor H.M.Gurling (Academic Department of Psychiatry, University College London Medical School, UK).

Out of the three assays, rs12613898 was proven to give non-interpretable results with all first 95 samples appearing heterozygotes for this SNP and therefore it was not performed on the remainder of the samples.

Genotyping results for rs1867846 and rs13028288 are shown in table 5.2 and the output of the statistical analysis is shown in table 5.3.

SNP	Samples	Genotype		
		Homozygote 1/1	Heterozygote 1/2	Homozygote 2/2
rs1867846	Controls	11	88	201
	Schizophrenics	8	92	197
rs13028288	Controls	221	70	3
	Schizophrenics	237	65	2

Table 5.2. *rs1867846and rs13028288 SNPs analysis in the London samples*

SNP	Tests for deviation from Hardy-Weinberg equilibrium		Tests for association (C.I.: 95% confidence interval)				
	Controls	SCZ	allele freq. difference	Heterozygous	Homozygous	Allele positivity	Armitage's trend test
rs1867846	n11=11 (10.08) n12=88 (89.83) n22=201 (200.08) f_a1=0.18 +/- 0.016 F=0.02041 p=0.723729 (Pearson) p=0.726036 (Llr) p=0.700868 (Exact)	n11=8 (9.82) n12=92 (88.36) n22=197 (198.82) f_a1=0.18 +/- 0.015 F=-0.04115 p=0.478197 (Pearson) p=0.469156 (Llr) p=0.562614 (Exact)	**Risk allele 2**				
			[1]<->[2]	[11]<->[12]	[11+]<->[22]	[11]<->[12+22]	common odds ratio
			Odds_ratio=1.010 C.I.=[0.753-1.355] chi2=0.00 p=0.94598 (P)	Odds_ratio=1.438 C.I.=[0.552-3.741] chi2=0.56 p=0.45524	Odds_ratio=1.348 C.I.=[0.531-3.421] chi2=0.40 p=0.52891	Odds_ratio=1.375 C.I.=[0.545-3.468] chi2=0.46 p=0.49827	Odds_ratio=1.045 chi2=0.00 p=0.94570
			Risk allele 1				
			[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	common odds ratio
			Odds_ratio=0.990 C.I.=[0.738-1.328] chi2=0.00 p=0.94598 (P)	Odds_ratio=1.067 C.I.=[0.750-1.517] chi2=0.13 p=0.71938	Odds_ratio=0.742 C.I.=[0.292-1.884] chi2=0.40 p=0.52891	Odds_ratio=1.031 C.I.=[0.733-1.448] chi2=0.03 p=0.86215	Odds_ratio=0.962 chi2=0.00 p=0.94570
rs13028288	n11=221 (222.91) n12=70 (66.18) n22=3 (4.91) f_a1=0.87 +/- 0.013 F=-0.05777 p=0.321894 (Pearson) p=0.294613 (Llr) p=0.440493 (Exact)	n11=237 (238.92) n12=65 (61.17) n22=2 (3.92) f_a1=0.89 +/- 0.012 F=-0.06262 p=0.274893 (Pearson) p=0.237724 (Llr) p=0.395936 (Exact)	**Risk allele 2**				
			[1]<->[2]	[11]<->[12]	[11+]<->[22]	[11]<->[12+22]	common odds ratio
			Odds_ratio=0.862 C.I.=[0.609-1.221] chi2=0.70 p=0.40369 (P)	Odds_ratio=0.866 C.I.=[0.590-1.271] chi2=0.54 p=0.46232	Odds_ratio=0.622 C.I.=[0.103-3.755] chi2=0.27 p=0.60114	Odds_ratio=0.856 C.I.=[0.586-1.250] chi2=0.65 p=0.42045	Odds_ratio=0.849 chi2=0.74 p=0.38921
			Risk allele 1				
			[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	common odds ratio
			Odds_ratio=1.160 C.I.=[0.819-1.642] chi2=0.70 p=0.40369 (P)	Odds_ratio=1.393 C.I.=[0.226-8.603] chi2=0.13 p=0.72022	Odds_ratio=1.609 C.I.=[0.266-9.717] chi2=0.27 p=0.60114	Odds_ratio=1.557 C.I.=[0.258-9.384] chi2=0.24 p=0.62646	Odds_ratio=1.181 chi2=0.74 p=0.38921

Table 5.3. *Hardy-Weinberg equilibrium and association analysis for rs1867846 and rs13028288 SNPs*

Further information on statistical analysis of rs1867846 and rs13028288 SNP genotyping can be found in Appendix D.

Statistical analysis of genotyping data for rs1867846 and rs13028288 confirmed that the distribution of the genotypes did not significantly deviate from the Hardy-Weinberg equilibrium, but this distribution was not significantly different between schizophrenic patients and controls. Therefore no association was found between any SNP of *KIAA1189* and schizophrenia in this population.

5.12- Discussion

The aim of the work described in this chapter was to confirm the EST AI07270 as a genuine rat transcript and to characterise it in relation with schizophrenia.

5.12.1- From the EST AI072720 to rat *RGD1308367*

When this work was initiated (in October 2003), the EST AI072720 was hardly annotated at any database and did not map to any rat gene. However, good sequence identity between the EST and the 3'UTR of human *KIAA1189* gene and with a rat genomic sequence downstream from the rat orthologue of *KIAA1189*, suggested that this EST may correspond to a rat transcript that had not been cloned or predicted yet. Better prediction and annotation of the human gene (than the rat gene) was not surprising since the first draft assembly of the human genome had already been announced (Lander *et al.* 2001; Venter *et al.* 2001) whereas the Rat Genome Sequencing Project was only under way.

Homology of the EST AI072720 sequence with a rat genomic sequence located downstream from a predicted gene was consistent with the fact that, like most ESTs, the EST AI072720 was 3'-derived and therefore likely to belong to an untranslated region lacking protein coding information. Although it would have been premature at this stage to rule out the possibility that the EST AI072720 was artefactual (such as being an unspliced cDNA, heterogeneous nuclear RNA or genomic DNA containing intragenic or intergenic sequences) (Wolfsberg and Landsman 1997), clustering of the EST AI072720 with three other ESTs provided evidence that the EST AI07270 did belong to a genuine transcript. Moreover, evidence showing that each of the four clustered ESTs had been generated from cDNA clones derived from brain (or eye) tissue suggested that this rat transcript may be, if not brain specific, at least predominantly expressed in the brain.

Interestingly, Unigene cluster Rn.20243 now includes 3 rat mRNA sequences (BC086596.1, NM_001008311.2 and DQ119821.1) and 32 ESTs (in total) which are all derived from cDNA libraries prepared from either specific brain regions or a

mixture of different brain regions, which confirms this hypothesis. This cluster is now identified as "RGD1308367: similar to KIAA1189 protein" which is consistent with our findings and with the current NetAffx annotation of rc_AI07270_at probe set as "similar to KIAA1189 protein (predicted)".

Numerous gene prediction programs were used to analyse a large piece of genomic sequence containing the EST AI072720, however, only a few of them (including GRAIL, GeneID and MZEF) predicted that the EST was part of a putative yet unpredicted exon. Interestingly, these programs were the ones which integrate multiple types of information including splice signal sensors, compositional properties of coding and non-coding DNA in order to predict entire gene structures (sets of spliceable exons) in genomic sequences (Burge and Karlin 1997). No other program suggested that the EST was part of the 3' untranslated region of the upstream *RGD1308367* gene. However, failure to determine the full extent of the transcript with its 3' extremity was not surprising since these gene prediction algorithms, although very accurate in identifying coding nucleotides, are still not reliable in grouping exons into transcripts (Burge and Karlin 1998; Claverie 1998). Moreover, prediction of a gene from this EST similarity search may have been affected by the presence of interspersed repeats (SINES, LINES, MAR etc ; Smit 1996) in the genomic sequence and the identification of exons or genes may have benefited from adequately filtering of these repeats to maintain the signal/noise ratio to an acceptable level (Claverie 1997). For these reasons, experimental work was required to determine the full extent of the transcript (5' and 3' ends of the gene) and of its alternative splice variants (Claverie 1997; Wu *et al.* 2004), particularly since the rat is one of the mammalian species whose sequence will not receive labour-intensive annotation efforts such as the ones devoted to human. However, another alternative approach that we may have used for identifying rat genes is the method described by Wu *et al.* (2004), who performed RT-PCR and direct sequencing based on dual-genome de novo predictions from TWINSCAN, a program which predicts which regions of a genome are transcribed into pre-messenger RNA, how they are spliced, and which portions of the spliced transcript are translated into protein (Korf *et al.* 2001; Flicek *et al.* 2003). This method, which was found to be cost-effective in confirming complete open reading frames and gene structures which were partially missed by the April 2003 Ensembl annotation of the rat genome, may well have been

effective in identifying the full-length transcript to which the EST AI072750 belonged.

5' RACE (rapid amplification of cDNA ends) PCR was performed to extend the EST sequence in the 5' direction and amplify the transcript to which this EST belonged. Amplification of the upstream sequence from the EST AI072720 produced a product of 3kb, suggesting that AI072720 was indeed the 3' end of *RGD1308367*. Aware of the technical difficulties of performing 5' RACE (Bashiardes and Lovett 2001), the experiment was repeated and again a 3kb RACE product was obtained. Unfortunately the yield of the amplicon was too low for sequencing. Therefore the hypothesis was further tested by predicting spliced products, which were amplified by PCR. These PCRs were performed using a proofreading enzyme (Kod Hot Start polymerase) designed for amplifying long targets with very high specificity and processivity and the longest predicted PCR product containing the 3 predicted exons of RGD1308367 and EST AI072720 was sequenced.

These experiments enabled confirmation 1) that the EST AI072720 was part of *RGD1308367* 3'UTR and 2) that *RGD1308367* included the three predicted exons. Failure to obtain the sequence of the 5' RACE product did not allow us to determine the full-length sequence of the transcript at the 5' end. Therefore *RGD1308367* full-length transcript (with its untranslated regions) may be longer than the 3kb product sequenced.

5.12.2- Validation of *RGD1308367* and *KIAA1189* differential expression

Differential expression of *RGD1308367* in the rat prefrontal cortex (PFC) after PCP treatment was confirmed by qRT-PCR: *RGD1308367* expression was significantly upregulated in the PCP model which is consistent with the microarray data from the chronic PCP model. In human post-mortem dorso-lateral prefrontal cortex (DLPFC), expression of *KIAA1189* (*RGD1308367* human orthologue) was significantly upregulated in a subset of our samples (n=5) corresponding to two populations of schizophrenic patients, namely the ones collected by Harvard and Brain-Net brain bank organisations. The direction of change was again consistent with that of the rat chronic PCP model of schizophrenia.

Differential expression of *KIAA1189* in some schizophrenic patients but not others may not be unexpected since the "schizophrenia" phenotype actually reflects a spectrum of disorders more than a single pathology and gathers together individuals with very different symptomatology. Moreover, independently of the potential correlation between the phenotype of these patients and their gene expression pattern, schizophrenia is also an etiologically complex disease whose mode of transmission is only compatible with a multigenic model in which no single gene can be assumed to contribute to schizophrenia in every affected individual or in any population (Gottesman and Shields 1967; McGue and Gottesman 1989; Owen *et al.* 2005a). Gene expression changes are therefore likely to be different between individuals so that they may not be detected when examining a set of clinically heterogeneous patients as one single group. In this present case, the difference in the origin of patients (Harvard, Brain-Net and UCLA brain banks) within the whole set of samples did add further heterogeneity to the set. Whereas there was not any obvious difference between patient characteristics (such as age, gender and post-mortem delay) or processing of samples across brain banks, lack of information about the criteria used by each organisation for diagnosing schizophrenia (and even the subjectivity of establishing this diagnosis) did not rule out the potential existence of major clinical differences between all the patients.

Differential expression of rat *RGD1308367* had previously been shown by qRT-PCR using an assay designed against the 3'UTR (EST AI072720, section 3.4.3). However, differential expression of the human gene, *KIAA1189*, was only shown when an assay in its coding region was used (section 5.4) but not when an assay in the 3'UTR was used (section 3.4.3). Indeed, although both human qRT-PCRs assays were examining the same gene, it is possible that *RGD1308367* may have alternative splice variants which may be specifically or differently detected by different qRT-PCR assays. Moreover, since probe sensitivity is a sequence-inherent characteristic, amplification efficiencies may be different between experiments which may lead to different results.

In situ hybridisation did not confirm differential upregulation of *RGD1308367* in the rat prefrontal cortex after chronic PCP treatment but revealed its significant decreased expression in two white matter regions, the fornix and the forceps minor of

the corpus callosum. Whereas the *RGD1308367* expression pattern looked consistently identical to the expression pattern of EST AI072720 (section 3.4.3), statistical analysis of the data yielded inconsistent results since no significant change had been found in the expression levels of EST AI072720 in any region of the rat brain after PCP treatment.

Apart from the issue of the inherent limited sensitivity of *in situ* hybridisation to detect low expression changes (section 3.5.4), these inconsistent results are likely to be due to differences between experiments, including the levels of cutting of the brains between the sets of sections used for the two studies. For example, the sections used for EST AI072720 were more posterior at the level of the thalamus than those used for *RGD1308367,* which reduced the size of the fornix for the analysis and prevented uniform detection of EST AI072720 expression within this region, making the two experiments impossible to compare. Moreover, another possibility in this context may be the expression of *RGD1308367* being slightly variable throughout the brain in a rostro-caudal manner, probably, in relation with variable density of neurons in these regions (Dombrowski *et al.* 2001), so that its differential expression in the rat chronic PCP model may appear at very specific levels only (as shown for other genes, Susan Cochran, unpublished observations). Since qRT-PCR and *in situ* hybridisation probes were designed to detect very close regions of the sequence, the issue of them detecting different splice variants, which may have explained both the failure to confirm the qRT-PCR results in the prefrontal cortex using *in situ* hybridisation and the differential expression shown in white matter regions using this technique, could be excluded. Differential downregulation of *RGD1308367* in some parts of the fornix and the forceps minor callosum after PCP treatment may therefore represent a genuine result which is worth analysing in relation with schizophrenia.

The fornix and the corpus callosum are key white-matter structures in the mammalian brain which ensure proper connectivity between brain structures and cerebral hemispheres respectively. Changes in gene expression, hence functional impairment, within such regions may therefore be consistent with the current hypothesis that schizophrenia is a disorder of cortical connectivity (Friston and Frith 1995; Friston 1998; Stephan *et al.* 2006).

The fornix is a bundle of axons which connects the hippocampus and the thalamus and has been shown to be involved in aspects of working memory (verbal and spatial working memory (Gaffan 1994; Calabrese *et al.* 1995; McMackin *et al.* 1995; Parker and Gaffan 1997) that are impaired in schizophrenic patients (Constantinidis and Wang 2004; Peuskens *et al.* 2005). Moreover, fimbria-fornix lesionned rats present a working memory impairment that is attenuated by treatment with the antipsychotic clozapine, which may reflect some of the cognitive performance improvements observed after clozapine treatment in people with malfunctions of the hippocampus such as seen in schizophrenia (Addy *et al.* 2005).

Although no alterations of the fornix have been reported to date in schizophrenia, both its function and the regions it connects (the thalamus and the hippocampus ; Andreasen 1997; Gothelf *et al.* 2000; Harrison and Weinberger 2005) have been implicated in schizophrenia. Consistently, decreased expression of *RGD1308367* mRNA in the rat fornix after PCP treatment may therefore be related to some aspects of working memory known to be impaired in schizophrenia.

The corpus callosum is the largest white matter structure in the mammalian brain. Its contralateral axon projections allow connectivity and communication between the left and right cerebral hemispheres and play an important role in the proper functioning of the prefrontal cortex (Carr and Sesack 1998). Hence, in view of the essential implication of the prefrontal cortex in schizophrenia pathophysiology, it may not be unexpected to observe decreases in white matter density in the corpus callosum, as was reported by Hushoff Pol *et al.* (2004). Besides, these changes were interestingly found to correlate to grey matter density changes and to illness severity (Hulshoff Pol *et al.* 2004) suggesting they are central to schizophrenia pathophysiology. Therefore, we propose that decreased expression of *RGD1308367* within the corpus callosum after PCP treatment may be related both to prefrontal activity and to aberrant connectivity in schizophrenia.

In summary, *RGD1308367 in situ* hybridisation revealed gene expression changes in white matter regions which may be particularly relevant to deficits in working memory and connectivity disturbances found in schizophrenia. A major caveat of this experiment is that the protocol used for this study did not have the power to determine the cellular localization of *RGD1308367* mRNA and therefore in which cell type the gene was expressed. Gene expression changes within white matter

regions may indeed relate to expression of *RGD1308367* within glial cells such as myelin-forming oligodendrocytes or within axonal projections of cortical (such as PFC) neurons suggesting that synthesis of RGD1308367 would occur in axons rather than in the cell body. In terms of function, both possibilities may be relevant to schizophrenia as altered myelination and abnormal refining of synaptic connectivity are the two developmental processes which have been proposed to be disturbed in schizophrenia leading to altered connectivity and dysfunction of the prefrontal cortex and triggering the emergence of symptoms and deficits during periadolescence or early adulthood (Woo and Crowell 2005). Interestingly, the hypothesis suggesting that *RGD1308367* may be translated locally in axons may be consistent not only with schizophrenia but also with the hypothesis that *RGD1308367* may have an ERM-related function as axonal protein synthesis has been shown to be involved in growth cone dynamics and axonal guidance (Zhang *et al.* 2001; Piper and Holt 2004).

Failure to detect or confirm significant alterations in *RGD1308367* expression after PCP treatment in cortical regions such as the prefrontal cortex is likely to be due to its very low expression levels within these regions combined with the limited sensitivity of this technique. In this context, more sophisticated techniques such as qRT-PCR on laser-microdissected tissue may be needed to detect significant changes in particular cortical regions. Such a study, performed using microdissected rat prelimbic cortex (Antonio Ferra and Catherine Winchester), failed to identify any significant changes in *RGD1308367* expression in this region after PCP treatment, suggesting that the increased expression of *RGD1308367* detected in the microarray did reflect a change in its expression in another part (infralimbic or ventral-orbital cortex) of the grossly dissected prefrontal cortex used in the microarray. However, this experiment confirmed that *RGD1308367* is expressed in the prelimbic cortex suggesting that it is definitely expressed by cortical neurons where it may have another role (potentially related to schizophrenia) than in the white matter.

Finally, since we failed to confirm the differential expression of *RGD1308367* in the prefrontal cortex after PCP treatment but showed expression changes within close white matter regions (the corpus callosum in particular), one could argue that the expression changes identified in the microarray did actually reflect expression changes within white matter tracts that would have been consistently dissected together with the prefrontal cortex. However, it is unlikely in that case that the same amount of white matter may be present in all samples, making possible the

identification of genes regulated in these regions. Conversely, the more likely differing amounts of white matter that may be due to inaccurate dissections would have affected the whole data set either by preventing the identification of differentially expressed genes because of heterogeneity or by "contaminating" the results with a number of myelin-related genes. This was not the case since this study did allow the identification of a reasonable number of differentially expressed genes, among which there were only very few myelin-related genes (Catherine Winchester), which refutes the hypothesis that the changes detected may reflect expression changes within white matter tracts rather than prefrontal cortical areas.

5.12.3- Genetic and genomic characterisation of *KIAA1189*

Mapping of human *KIAA1189* gene to chromosome 2q24.1 is very unlikely to be irrelevant to schizophrenia since it is very close to the only region (chromosome 2p12-q22.1) which reached genome-wide significance in a meta-analysis including 20 individual genome scans (Lewis *et al.* 2003). Loci spanning from 2q11 to 2q37 have been linked with schizophrenia in five independent genome scans (Levinson *et al.* 1998; Williams *et al.* 1999; Mowry *et al.* 2000; DeLisi *et al.* 2002a; DeLisi *et al.* 2002b) (Wijsman *et al.* 2003) and chromosome 2q36 has recently been associated with visual working memory in a linkage study looking at cognitive trait components of schizophrenia (Paunio *et al.* 2004).

All these data suggest that chromosome 2q (including 2q24.1) is not just one of the many loci identified across the genome but rather one of the most well supported loci linked with schizophrenia. Mapping of *KIAA1189* within this region therefore provides strong genetic evidence suggesting that this gene may account for some susceptibility to schizophrenia and supports the hypothesis of *KIAA1189* being an interesting candidate gene to investigate in relation to this disease.

Failure to detect any significant association between *KIAA1189* and schizophrenia in a population of 600 schizophrenic patients and controls (n=300) did not provide any additional evidence of a potential role of KIAA1189 in schizophrenia. However, this study was quite preliminary in terms of design and more sophisticated analysis may be necessary for increasing the likelihood of identifying a significant genetic association. In particular, since schizophrenia displays considerable heterogeneity of

symptoms which may reflect several different disease processes (Owen *et al.* 2005a), it may be very beneficial to refine the phenotype used for this study by defining sub-groups of patients based on intermediate endophenotypic measures (Leboyer *et al.* 1998; Gottesman and Gould 2003). Indeed, such endophenotypes, which occur across the schizophrenia spectrum in schizophrenia patients, may be more amenable to quantitative genetic analyses and some of them may even be conceptualised as being impaired because of a single genetic abnormality (Braff and Light 2005). Drug responsiveness may also be a parameter that could be used for defining sub-groups of patients and increase the likelihood to get a significant association by minimising sample heterogeneity. Increasing sample size may also be needed as schizophrenia is a complex disorder whose genetic component is likely to involve a few or several genes which may contribute to the susceptibility for schizophrenia, so that larger samples may be required to reliably detect significant associations of single genes with schizophrenia. Moreover, independently of the sample size and of the phenotype used, another way to increase the power of this study may be to examine haplotypes instead of considering each SNP separately. Usage of haplotypes has indeed been shown to increase the power of genetic association studies by reducing the dimension of statistical tests for association (Clark 2004; de Bakker *et al.* 2005). Therefore to maximise the power of the analysis, association studies should ideally be designed to take into account all these different parameters. However, this is quite a recent concept and failure to detect or to replicate significant genetic associations in complex diseases using designs similar to that used here has long been very common in the literature. Thus, it would be very premature to draw any conclusions from this study and, on that assumption, to refute the hypothesis that *KIAA1189* may have a role in schizophrenia.

5.12.4- *In silico* analyses of *KIAA1189* expression and function

KIAA1189 tissue expression profile was obtained from different sources, including GeneNote (Shmueli *et al.* 2003) and Unigene (Pontius 2003) which consist respectively of a database of gene expression in twelve normal adult human tissues based on in-house DNA array experiments using Affymetrix GeneChip HG-U95A-E, and of a reconstruction of gene expression profiles from unbiased cDNA library data based on the frequency of occurrence of an EST in Unigene EST clusters.

Information about KIAA1189 expression (RT-PCR and ELISA data) was also obtained from the Kazusa DNA Research Institute which has made fundamental information on the structure and function of genes available to the public. Very interestingly, all three databases consistently showed that *KIAA1189* is highly expressed in the brain (including both white and grey matter regions) and in the spinal cord in comparison with other tissues of the body, which was consistent with the hypothesis that *KIAA1189* may have a role in schizophrenia pathophysiology.
In addition, further support for the potential involvement of KIAA1189 in schizophrenia was provided by data showing that AI207881, one of the many human ESTs which have been attributed to KIAA1189, was first identified as expressed in the frontal lobe of a schizophrenic patient (Mieg *et al.* 2005).

Finally, RGD1308367 and KIAA1189 putative proteins were investigated for the presence of typical domains which may give an insight into the potential role of the rat or the human protein. Both amino acid sequences were found to have limited similarity with the ERM (ezrin-radixin-moesin) proteins, which typically contain an N-terminal FERM (4.1, ezrin, radixin, moesin) domain which is known to bind membrane proteins followed by a long helical coiled-coil region and a C-terminal filamenteous (F)-actin binding site (Bretscher *et al.* 2002; Ramesh 2004). ERM proteins are ubiquitously expressed proteins which cross-link membrane proteins to the cortical cytoskeleton and are involved in the regulation of the actin cytoskeleton (Ramesh 2004). RGD1308367 and KIAA1189 both lack the N-terminal FERM domain but show a weak similarity along the α-helical central region and shared 18 almost identical amino acids with ERM proteins in their F-actin binding site (Bretscher *et al.* 2002; Ramesh 2004). KIAA1189 may therefore be considered as a distant member of the ERM family of proteins which may bind F-actin but not membrane proteins. Thus, limited similarity with ERM proteins was not enough evidence to provide an insight into KIAA1189 function, which may or may not be related to that of ERM proteins. However, since the activity of ERM proteins has been shown to be regulated by intramolecular associations between their N-terminal and C-terminal domains (Louvet-Vallee 2000), one hypothesis could be that KIAA1189 may function as an endogenous regulator of ERM proteins by interacting though its C-terminal domain with the N-terminal domain of ERM proteins thereby further regulating their activity. Interestingly, such a function may be relevant to

schizophrenia as ERM proteins, via their interaction with the actin cytoskeleton, regulate the development and the maintenance of growth cones, these highly dynamic structures which guide the developing neurites to their distant targets and are involved both in their elongation and branching (Goldberg and Burmeister 1989; Devoto 1990; Dent *et al.* 2003). Thus, a potential role of KIAA1189 in the regulation of the function of ERM proteins hence in growth cone dynamics may be consistent with a number of abnormalities that have been reported in schizophrenia including abnormalities in synaptic and dendritic arborisation as well as reduced volume of neuropil (Lewis 1997; Selemon and Goldman-Rakic 1999) and altered rearrangement of neuronal connections (Benes 1995; Benes 1997).

However, independently of this potential role of KIAA1189 in the regulation of ERM protein function, binding of KIAA1189 to F-actin via its C-terminal ERM domain may still be consistent with many cytoskeletal abnormalities found in schizophrenia. Indeed, actin, as a major part of the cytoskeleton, has been shown to play a major role in the morphological development of neurons and in the structural changes of adult neurons (Luo 2002). In particular, actin plays a critical role in the formation and plasticity of dendritic spines, whose density has been shown to be reduced in neurons of the hippocampal formation and dorso-lateral prefrontal cortex in post-mortem studies of schizophrenic patients (Glantz and Lewis 2000; Kolluri *et al.* 2005; Kolomeets *et al.* 2005). Moreover, the integrity of the actin cytoskeleton is a determinant for the activity of NMDA receptors and activation of NMDA receptors in turn initiates changes in the actin cytoskeleton of dendritic spines that stabilise synaptic structure (Ackermann and Matus 2003). Thus, alterations of actin cytoskeleton or disruption of its dynamics during development or in response to learning and experience may also be consistent with the NMDA receptor hypofunction in schizophrenia (Coyle *et al.* 2003).

5.12.5- Functional characterisation of KIAA1189 *in vitro*

Unfortunately, although the FLAG® epitope has been shown to facilitate superior detection and purification of recombinant fusion proteins when using highly specific and sensitive anti-FLAG® antibodies, we failed to confirm the direct interaction between KIAA1189 and actin by FLAG® immunoprecipitation in C6 cells

transfected with the FLAG®-KIAA1189 construct. Because the FLAG® epitope was expressed at the N- rather than at the C-terminus of KIAA1189 (where the protein harbours its ERM-like actin-binding site), it is unlikely that its presence affected the function of the protein. However, failure to detect any of its interacting partners may not be unsurprising if KIAA1189 is an ERM protein since the self-associating activity of these proteins has been shown to interfere with their association with other binding partners thereby preventing their identification using this technique (Vaheri *et al.* 1997; Bretscher *et al.* 2002). Similarly, following on the hypothesis (see above) that the C-terminal domain of KIAA1189 may bind the N-terminal domain of ERM proteins, KIAA1189 could be suggested to form a regulatory complex with ERM proteins in which the FLAG® epitope would be masked. Yet another hypothesis is that coimmunoprecipitation of the protein and its partners failed because the overexpressed KIAA1189 proteins were not functional. Indeed, although a FLAG®-KIAA1189 fusion protein of about the right size had been shown to be translated from the transfected construct using rat C6 cells, we cannot exclude that this protein may have small structural defects, may have undergone misfolding or may need to be activated to become functional. Indeed, the KIAA1189 amino acid sequence suggests many potential phosphorylation sites including protein kinase C, cAMP-dependent protein kinase and serine/threonine/tyrosine phosphorylation sites. In particular, KIAA1189 was predicted to have a threonine phosphorylation site at its C-terminus which may correspond to a specific residue of ERM proteins (Simons *et al.* 1998) which was shown to contribute to their activation by unmasking of their F-actin binding site, independently of their N-terminal ERM site. Therefore phosphorylation of this particular site and/or others is likely to be necessary to activate the protein and reveal its potential actin-binding function. Finally, independently of all proposed hypotheses, it may be also be that the cell line used was not suitable for examining KIAA1189 function. Indeed, rat C6 glioma cells were used as a model of glial cells but we did not have any direct evidence that KIAA1189 is expressed in glial rather than neuronal cells (see discussion about *in situ* hybridisation results). Therefore it may well be that KIAA1189 was not functional in these cells. Further experiments would have been needed using different cell lines as well as different potential activators of this protein in order to identify its potential interacting partners.

A nice way to refine the hypothesis would have been to examine the subcellular localisation of endogenous RGD1308367 protein in the rat brain and to investigate

potential protein expression changes in the rat chronic PCP model. Unfortunately, commercial antibodies were not available for RGD1308367 or KIAA1189 (due to time constraints we were unable to generate our own) so that we decided to investigate KIAA1189 function *in vitro* by overexpressing in mammalian cells. Since failure to identify any potential KIAA1189 interacting partners by coimmunoprecipitation using C6 cells may be explained by many issues, we decided to investigate KIAA1188 function not only in these cells but also in more neuronal-like cells. Rat C6 glioma cells, which were originally cloned from a chemically induced rat brain tumour and classified as an undifferentiated astrocytic cell type (Benda *et al.* 1968), were thus used to investigate KIAA1189 function in glial-like cells while the rat PC12 cell line, established from a transplantable rat adrenal pheochromocytoma and which responds reversibly to nerve growth factor (NGF) by induction of a neuronal phenotype (Greene and Tischler 1976) was used as an *in vitro* model for the study of the role of KIAA1189 in neuronal-like cells. Again, KIAA1189 was expressed as a fusion protein with an N-terminal FLAG® to allow immunocytochemistry studies through FLAG® immunoreactivity.

In C6 glioma cells, KIAA1189 was found to be predominantly expressed within the plasma membrane (or very close to it) and within neuronal-like processes, showing an expression pattern which at first appeared very similar to that of actin. However, thorough investigation of colocalisation of KIAA1189 with actin revealed that the proteins only colocalised at very few sites, not providing enough evidence to confirm KIAA1189 interaction with actin. KIAA1189-transfected cells did not present any striking phenotypic difference compared with C6 cells expressing the empty FLAG® expression vector which did not allow us to draw any hypothesis on KIAA1189 function in these cells.

Keeping in mind that there may be an activation issue, KIAA1189 was overexpressed in rat PC12 cells to study its function in neuronal-like cells as opposed to glial-like C6 cells. In differentiated PC12 cells, KIAA1189 was found to be predominantly expressed in neurites and in the plasma membrane which was consistent with the expression of ERM proteins at the cytoplasmic surface of plasma membranes (Sato *et al.* 1991). KIAA1189 expression pattern did not overlap exactly with that of actin suggesting that it did not bind actin throughout out the cell body as a structural element of the cellular cytoskeleton. However, KIAA1189 was found to

colocalise with actin in some processes and especially at the end of some neurite tips as well as in the plasma membrane around the cell body.

Colocalisation of KIAA1189 and actin in the plasma membrane suggested that KIAA1189 may bind actin at cell membrane-actin adhesion sites. Although KIAA1189 was not predicted to bind membrane proteins, this expression pattern was therefore consistent with that of ERM proteins at cell-surface structures (Ramesh 2004). High expression of KIAA1189 in neuronal processes and at the end of neurite tips suggested that KIAA1189 may have a role in the architecture and in the extension of neurites, which was consistent with its partial colocalisation with actin at some of these sites which are critical in neurite outgrowth. These studies therefore provided evidence suggesting that KIAA1189 may be related to ERM proteins which are known to provide a link between membrane proteins and the cytoskeleton and to regulate growth cone motility in neurons by interacting with F-actin (Ramesh 2004).

In the context of our *in situ* hybridisation results, predominant expression of KIAA1189 within neuronal processes may be consistent with the hypothesis according to which *RGD1308367* mRNA expression within white matter tracts did actually reflect its presence within the axons of cortical neurons, where local protein synthesis would have occurred. Such an axonal translation, which is much more flexible and efficient than axoplasmic transport, has been suggested to provide a rapid supply of proteins at the synapse thereby playing an essential role in modulating synaptic plasticity and long-term potentiation (Piper and Holt 2004). Alterations in axonal translation of critical proteins may thus be consistent with the hypothesis of schizophrenia being a "disorder of the synapse" (Owen *et al.* 2005b) by providing an explanation and a molecular substrate to the altered plasticity (altered response to guidance clues and long-term potentiation) in schizophrenia (Stephan *et al.* 2006). Such abnormalities have besides already been suggested in neuronal-based diseases such as spinal muscular atrophy (Jablonka *et al.* 2004) and Fragile X syndrome (Antar and Bassell 2003).

Both the KIAA1189 expression pattern and its partial colocalisation with actin in differentiated PC12 cells suggested that this protein may have a role in neurite extension.

Semi-quantitative measurement of neurite outgrowth using PC12 cells showed that KIAA1189 overexpression prior to differentiation prevented the extension of neurites

by comparison with PC12 cells transfected with the empty expression vector. This finding was confirmed by looking at cell morphology in comparing GFP epifluorescence of FLAG®-KIAA1189/GFP and FLAG®/GFP co-transfected PC12 cells: KIAA1189 overexpression was associated with shorter neurites. Although further experiments would be needed to exclude the possibility that the observed reduced neurite length shown by FLAG®-KIAA11889 transfected cells may reflect neurite retraction during cell death rather than genuine alterations in neurite extension due to FLAG®-KIAA1189 overexpression, no obvious signs of apoptotic cell death (nuclear condensation and fragmentation) were observed in these cells. This strongly suggested that KIAA1189 did alter normal processes of neurite outgrowth in neuronal-like cells, as it indeed has been shown for mutant DISC1 protein (Ozeki *et al.* 2003). Interestingly, these results were also consistent with the upregulation of RGD1308367 expression in the rat prefrontal cortex after PCP, in the sense that increased expression could be linked with impaired synapse formation.
This role of KIAA1189 appeared reminiscent of the ability of other ERM proteins to affect cell morphology (Martin *et al.* 1997; Litman *et al.* 2000). Moreover, it was consistent with the hypothesis that the C-termini of ERM proteins may be involved in the organisation of the cytoskeleton at particular cortical sites and may modulate membrane protrusive activity at those sites (Henry *et al.* 1995).

All together, these results suggested that KIAA1189 may somehow interact (directly or indirectly) with actin thereby affecting the tightly-regulated cytoskeletal dynamics, which are essential for neurons to extend their processes and steer them to their potential synaptic partners (Luo 2000). As it is the interplay between cytoskeletal elements (especially microtubules and actin) (Jan and Jan 2003) which governs growth cone motility and neurite outgrowth, many different mechanisms could be proposed by which KIAA1189 may disrupt or dysregulate actin dynamics leading to alterations in neuronal cell morphology. However, in terms of temporality, the suggestion that FLAG®-KIAA1189-overexpressing cells presented shorter rather than fewer neurites provided evidence indicating that KIAA1189 may be involved in the process of neurite extension rather than in the initial steps of neurite induction (formation of protusions) and sprouting.

5.12.6- A potential role for KIAA1189 in schizophrenia

Alterations in neurite outgrowth are consistent with the neurodevelopmental hypothesis of schizophrenia, which suggests that schizophrenia results from a disturbance in a maturational or developmental brain process (Weinberger 1987; Harrison 1999; Andreasen 2000; Lewis and Levitt 2002; Sawa and Snyder 2002). Recent studies have provided reliable evidence in favour of this hypothesis and converge on suggesting that cytoskeletal abnormalities (such as alterations of neuronal size and synaptic and dendritic organisation) actually reflect subtle alterations in schizophrenic cerebral cortical development which may become apparent at critical periods of the development such as the onset of puberty, and may explain at least a proportion of the defective synaptic plasticity and connectivity observed in schizophrenia. These alterations, which are likely to concern the basic steps of brain histogenesis (cell production, migration, neurite outgrowth and formation of the synaptic circuitry) may be, at least in part, associated with actin dynamics (Kamiya *et al.* 2005).

By affecting neurite extension *via* some disruption or dysregulation of cytoskeletal dynamics, probably at a critical time during brain development, KIAA1189 may thus play a critical role in the development of the cerebral cortex so that subtle alterations in its expression may lead to abnormalities in neuronal connectivity which may contribute to schizophrenia pathogenesis (Sawa and Snyder 2002).

However, with regards to the chronic PCP model, a slightly different hypothesis has to be proposed since changes in RGD1308367 expression in this model were induced by chronic administration of PCP to adult (~3 month-old) rats in which all brain regions, even the prefrontal cortex (the latest brain region to achieve maturation) were likely to be mature already. Therefore a potential contribution of RGD1308367 to alterations in the development of the cerebral cortex cannot explain the phenotypic range of metabolic, neurochemical and behavioural changes observed in PCP-treated rats (Morris *et al.* 2005). Rather, KIAA1189 may play a role in the plasticity of the brain in response to learning and experience so that its altered expression may contribute to abnormal pruning of synapses during adolescence and early childhood and to impaired neurotransmission in adults, both of which have been proposed in schizophrenia (Harrison 1999; Rapoport *et al.* 2005; Stephan *et al.* 2006). In particular, KIAA1189 may be involved in the chemical and structural modifications

of dendritic spines which are known to be essential for synaptic plasticity (Hering and Sheng 2001; Yuste and Bonhoeffer 2001) and whose development and stability is highly dependent on the actin cytoskeleton (Fischer *et al.* 1998; Luo 2002). This hypothesis would therefore be consistent with the number of alterations in dendritic spines which have been shown in the dorso-lateral prefrontal cortex of schizophrenic patients (Carr *et al.* 1999; Flores *et al.* 2005; Hill *et al.* 2006).
Moreover, this proposition may also explain the ability of PCP to induce a psychosis in adult humans which closely resembles schizophrenia and is representative of not only the negative and positive symptoms of the disease but also the cognitive deficits (Adler *et al.* 1999). At the molecular level, this effect of PCP may be related to its NMDA antagonism properties since NMDA receptor activity, which has been shown to be highly interdependent on the actin cytoskeleton, is essential for long-term potentiation and synaptic plasticity (Carlisle and Kennedy 2005).

We suggest as a general hypothesis that KIAA1189 may affect the assembly and/or disassembly of actin filaments in extending neurites during development by interacting with actin-binding proteins or by direct interaction with actin, or be involved in the rapid reorganisation of the actin cytoskeleton necessary for neurite outgrowth. As actin turnover in the growth cone has been shown to be tightly regulated as the brain matures, its dysregulation at critical developmental stages is likely to induce important abnormalities in neuronal cytoarchitecture. In adults, KIAA1189 is likely to play a role in synaptic plasticity by mediating dendritic spine changes in response to experience and learning.
Whether KIAA1189 directly interacts or not with actin (and how) will have to be determined to elaborate a more precise hypothesis on KIAA1189 functional role in neuronal architecture and neurite outgrowth. *In vitro* studies may be performed first to determine if KIAA1189 binds to filaments or monomers of actin, whether it bundles F-actin and investigate the effects of the protein on actin polymerisation.

While this study was close to completion, KIAA1189, under the name of "Ermin", was reported in the literature and characterised as a myelinating oligodendrocyte-specific protein (Brockschnieder *et al.* 2006). Ermin (another name of KIAA1189) had been identified as exclusively expressed in oligodendrocytes in a microarray comparing cDNAs from wild-type and dysmyelinated mice. An antibody was raised

to examine the endogenous expression of the protein in the brain and in cell cultures. Consistently with our *in situ* hybridisation results, they found that Ermin is expressed along white matter tracts and claim that it is not expressed by astrocytes or neurons. However, these data are not shown in their article which may cast some doubt on the clarity of their findings and may leave room for our hypothesis of neuronal expression of KIAA1189. Consistent with this, a multiple tissue northern blot shows low but genuine expression of *Ermin* in cortical areas, suggesting that this protein may have a role there. To examine this hypothesis further, qRT-PCR was performed on laser-capture microdissected prelimbic cortex, where the level of *RGD1308367* (rat orthologue of *KIAA1189*) was high enough to quantify easily (Antonio Ferra and Catherine Winchester). Although this dissected part of the prefrontal cortex did necessarily represent a heterogeneous population of neuronal and glial cells, it appeared unlikely that this expression of *RGD1308367* in a region of the cortex where the ratio of glia/neurons is around 0.9 (which theoretically means that there are a few more neurons than glial cells in this region, (Dombrowski *et al.* 2001), only reflected glial expression. Consistently with our hypothesis, these data therefore further suggested that *RGD1308367* was genuinely expressed in neurons.

As soon as in August 2005, another paper was also published that described the identification of a novel oligodendrogial protain, called juxtanodin (Zhang *et al.* 2005a), but the relevance of this gene to this work was not fully realised until its completion. Juxtanodin, which had been identified during a process of screening cell-type-specific CNS genes, appears indeed very similar to RGD1308367 in terms of sequence and very similar to Ermin in terms of functions. In their study, Zhang *et al.* (2005) showed that at the cellular level juxtanodin colocalises with 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase), a cytoskeletal-related oligodendroglial protein, and that in the myelin sheath its expression delineates the juxtanode, at the Ranvier node/paranode junction (Zhang *et al.* 2005a). However, the authors also describe expression of juxtanodin. With regards to its role, juxtanodin was found to promote arborisation of cultured oligodendroglia cells and increase transport to the process arbors of oligodendrocyte precursors. Therefore the authors suggest that juxtanodin is a cytoskeleton-related oligodendroglial protein that may be involved in CNS myelination and/or specialisation at the node of Ranvier (Zhang *et al.* 2005a).

At present and after performing a number of alignments between RGD1308367 and juxtanodin mRNA, it appears that these different names are in fact likely to relate to the same gene and therefore to Ermin as well, which was shown to be likely to represent the same gene as RGD13098367. The identity of Ermin and Juxtanodin were checked and the GeneBank accession numbers and GeneIDs reported in the different papers were confirmed to correspond to that of rat RGD1308367 and human KIAA1189 mRNAs and proteins. However, Brockschnieder *et al.* (2006) cloned the open reading frame of Ermin from mouse brain by PCR and Zhang *et al.* (2005) cloned fragments of a rat library cDNA clone, while we cloned a commercial Image Clone of human KIAA1189 cDNA. The sequences were confirmed to match database sequences and were therefore likely to correspond to genuine orthologues. However, we cannot ascertain that they are in fact genuine orthologues and that both the cDNA probes and the antibody Brockschnieder *et al.* (2006) designed against the mRNA and the protein were specific to this transcript and did not detect any other potential (although not predicted) splice variant of this gene or even any very similar transcript.

Nevertheless, evidence showing that Ermin is a marker of myelinating oligodendroglia and plays a role in cytoskeletal rearrangements during myelination (Brockschnieder *et al.* 2006), as well as evidence showing that juxtanodin might be involved in CNS myelination and might play a role in oligodendrocyte motility, differentiation or myelin-axon signalling, may be consistent with published literature showing oligodendrocyte dysfunction and altered myelination in schizophrenia (Hakak *et al.* 2001; Pongrac *et al.* 2002; Davis *et al.* 2003; Lehrmann *et al.* 2003; Tkachev *et al.* 2003). Interestingly, such a hypothesis would make the *in situ* results showing decreased expression of RGD1308367 in white matter regions after PCP treatment relevant to oligodendrocyte dysfunction and altered myelination in schizophrenia.

Therefore the results presented here are completely compatible with those of Brockschnieder *et al.* (2006) and Zhang *et al.* (2005) and suggest that Ermin/Juxtanodin/KIAA1189 is likely to have dual functions in oligodendrocytes and neurons, both of which may be related to schizophrenia.

In summary, the work described in this chapter provides several lines of evidence which suggest that KIAA1189 may be involved in schizophrenia susceptibility and pathophysiology: 1) KIAA1189 maps to a key schizophrenia locus (genetic evidence), 2) KIAA1189 expression is predominant in the brain by comparison with other tissues and its expression was altered both in the rat prefrontal cortex after PCP treatment and in human post-mortem dorso-lateral prefrontal cortex of schizophrenic patients (genomic evidence) and 3) KIAA1189 may interact with actin at the plasma membrane and may play a critical role in neurite extension and synaptic plasticity in the brain which is consistent with schizophrenia pathophysiology and hypotheses (functional evidence).

KIAA1189 therefore appears as a completely novel and intriguing candidate gene for schizophrenia.

CHAPTER 6: SUMMARY AND FINAL DISCUSSION

The overall aim of this work was to identify and characterise novel genes that may be associated with schizophrenia and may be particularly relevant to the development of novel antipsychotic drug treatments for the negative and cognitive symptoms of schizophrenia.

The rat chronic phencyclidine (PCP) model developed in YRING was used to this aim as it provides a very valuable model of the schizophrenia syndrome, displaying a pattern of metabolic, neurochemical and behavioural deficits that closely mirror that observed in schizophrenic patients. In addition, the ability of this model to very reliably model the cognitive deficits observed in schizophrenia is particularly relevant to the development of antipsychotic compounds since improving atypical activity is probably the major challenge (together with improving drug safety and reducing the number of side effects of antipsychotics) the pharmaceutical industry has to face at present.

Microarray analysis of gene expression in the prefrontal cortex of the rat chronic PCP model of schizophrenia was utilised as a cutting-edge technology of assessing the transcriptome profile in this given brain tissue as a reflection of gene expression in a key brain region involved in schizophrenia. Thus, this study may compare to the number of microarray analyses of gene expression profiles that have recently been successfully applied to the study of central nervous system (CNS) diseases, in particular schizophrenia (Mirnics *et al.* 2006). However, beyond the issue of using an animal model of a disease and potentially comparing data obtained using this approach with results obtained using post-mortem brain tissue from affected individuals, there is such a discrepancy between experimental designs across studies that it would be unreasonable to compare the data obtained in our study and the genes identified as differentially expressed in our conditions with other schizophrenia microarray results. Thus, in addition to the differences in the studied cohorts or tissue samples, usage of different microarray platforms and varying statistical analysis of microarray data are two of the factors whose influence on the outcome of microarrays has largely been studied and is likely to explain a significant proportion of the divergent profiles of gene expression yielded by different microarray studies (Tan *et al.* 2003; Hollingshead *et al.* 2005). The lists of differentially expressed genes across platforms show indeed relatively little overlap, as do the fold changes of potential differentially expressed genes concomitantly identified in different studies (Yuen *et al.* 2002a). Nevertheless, rather than

suggesting that microarray experiments are useless, these findings, in particular the confirmation of the potential involvement in schizophrenia of a few genes identified in these but not other microarray experiments, suggest that microarray analyses conducted to date may provide incomplete data and will therefore benefit from further evolution of microarray analysis tools and improvements in platform sensitivity (Mirnics *et al.* 2006).

In this context and although validation of small gene expression changes may be challenging (section 3.5.3), data verification by independent methods appears a critical step that has to remain a major part of transcriptome profiling experiments (Mimmack *et al.* 2004). As it was practically confirmed in this work (with *Tm4sf12*, section 3.4.2), usage of such independent methods of assessing gene expression may indeed be particularly very valuable in identifying false positives (type I errors) necessarily present among all identified differentially expressed genes, while false negatives (type II errors) should not be considered a definite proof of unaltered gene expression.

6.1- A few novel candidate genes for schizophrenia

Microarray analysis of gene expression in the prefrontal cortex of rats chronically treated with PCP revealed the differential expression of a number of ESTs whose potential to represent novel and uncharacterised genes or transcripts was exploited by a thorough manual bioinformatics analysis process. Thus, individual homology screening of the 209 identified ESTs against genomic and cDNA databases across species (rat, mouse, human) revealed 66 cDNAs, a proportion which further supported the value of our strategy to genuinely identify differentially expressed uncharacterised genes and suggested that the list of identified genes was unlikely to be exhaustive.

Nevertheless, as genuine as the differential expression of these genes may be, whether it reflects causative or adaptational changes to schizophrenia cannot be determined straightforwardly. Different classes of transcriptome changes can indeed be expected with regards to the mechanism of altered gene expression in complex brain disorders and they are not easy to separate. Thus, even if the use of a rat animal

model of the disease allows the exclusion of the possibility that gene expression changes may be related to an additional treatment (which could be the case in human schizophrenic patients), these changes may still represent primary or secondary changes, that is, they may either reflect the core causative basis whereby chronic PCP treatment triggers schizophrenia-like symptoms, or, they may reflect potential adaptations developed by rats following their enduring schizophrenia-like symptoms induced by chronic PCP treatment. Obtaining conclusive information on causality between gene expression and behavioural phenotype is very challenging and cannot be obtained using a single approach, however this issue may not matter much in terms of drug development in this context since all the genes which are differentially expressed in the chronic PCP model are likely to converge in "disease-affected molecular hubs" (Mirnics *et al.* 2006) that may represent correlates of the behavioural features of this model. Analysing gene expression changes in the brain of schizophrenic patients would be a very different situation, since expression changes may then also arise from a number of genetic susceptibilities and environmental insults that may be much more specific to individuals or subsets of individuals and therefore would not constitute promising drug targets for most patients with schizophrenia.

The potential significance of the 66 identified differentially expressed genes with relation to schizophrenia was assessed by doing some data mining on each of these genes. Although this process was conducted very thoroughly using a few criteria, the idea was really to get an overall picture of each gene in terms of function (known or predicted) and potential association with schizophrenia, in order to be able to make an informed decision on the potential value of each gene to constitute the basis of novel hypotheses of schizophrenia pathophysiology and/or to potentially represent promising drug targets for the treatment of schizophrenia. Thus, the rationale for the selection of *Edg2*, *Tm4sf12* and the EST AI072720 was very different, each of these candidates being typically taken up at different stages of characterisation and association with schizophrenia: *Edg2*, a G-protein coupled receptor with high affinity for lysophosphatidic acid (LPA), had indeed already been linked with schizophrenic-like behaviour (Harrison *et al,* 2003) while *Tm4sf12* (*tetraspanin 12*) had been suggested to be involved in potentially schizophrenia-relevant oligodendrocytes signalling, and the EST AI072720 may or may not correspond to a genuine rat

transcript predicted to possess an ezrin-radixin-moesin (ERM) domain that may be functionally relevant to schizophrenia. Consistent with the divergence of findings across microarray studies of schizophrenia, none of these genes had been previously identified in gene expression profiling of brain tissue either from any animal model of schizophrenia or from post-mortem tissue from schizophrenic patients. Only *Edg2* had been previously detected in other contexts since it was shown to be differentially expressed in the temporal cortex from patients with major depressive disorder (Aston *et al.* 2005) and in the nucleus accumbens of corticotropin releasing factor (CRF) overexpressing mice (Peeters *et al.* 2004). Interestingly, differential expression of *EDG2* as a myelination-related gene in major depressive disorder was suggested to be part of potential common oligodendroglial abnormalities between this disorder and schizophrenia and bipolar disorder (Aston *et al.* 2005). In addition, although it was not a microarray experiment, evidence has been published showing differential expression of LPA acyltransferase, *i.e.* the enzyme that catalyses the conversion of lysophosphatidic acid (LPA) to phosphatidic acid (PA), in the mouse striatum and frontal cortex in response to clozapine and haloperidol treatment, suggesting that LPA metabolism as part of the cell lipid metabolism pathways may be of importance in the mechanisms of antipsychotic drug action and in the pathophysiology of psychiatric disorders (Thomas *et al.* 2003).

As there is no consensus concerning microarray data analysis and because of the number of factors that affect and complicate brain disease microarray results, independent validation of the differential expression of the 3 selected candidates was undertaken as a critical prerequisite to further characterisation of these genes.

Success in confirming the differential expression of *Edg2* and EST AI072720 by qRT-PCR in the rat chronic PCP model despite the low fold changes they exhibited provided evidence not only of the sensitivity of the technique but of the reliability of the chronic PCP model to exhibit consistent gene expression patterns. Hence, failure to confirm the differential expression of *Tm4sf12* in the rat chronic PCP model using this technique provided further evidence of the necessity to validate microarray results. In the context of this work, because confirmation of microarray changes was strictly considered a prerequisite for further characterisation, *Tm4sf12* was not investigated further. Nevertheless, confirmation of such low fold changes is so difficult (section 3.5.3) that *Tm4sf12*, whose function may be relevant to

schizophrenia, may well not be a false positive in the microarray but a genuinely differentially expressed gene whose differential expression, by coincidence, was not confirmed in the rat chronic PCP model and in post-mortem tissue from schizophrenic patients. Conversely, *Edg2* and EST AI072720 were taken forward for further genetic, genomic and *in vitro* functional characterisation studies

6.2- EDG2, a promising drug target for schizophrenia?

EDG2 (also referred to as LPA1) is a G-protein-coupled receptor for lysophosphatidic acid (LPA), predominantly expressed in the brain, where its temporal expression and its expression pattern in the adult were suggested to reflect a potential role in neurodevelopment and/or in the control of myelination, both functions potentially relevant to the aetiology of several psychiatric disorders including schizophrenia (section 1.1.5). Consistent with this hypothesis, *Edg2* knock-out mice were shown to exhibit deficits in prepulse inhibition (PPI) and neurochemical changes that have been associated with a schizophrenic-like pathology (Harrison *et al.* 2003; Roberts *et al.* 2005). However, whether this potential role of Edg2 in schizophrenia was related to its expression in the white matter remains to be confirmed. Evidence of the role of Edg2 in interneuronal function and interneuron-mediated brain rhythms (Cunningham *et al.* 2006) may indeed provide another hypothesis whereby alterations in neuronal expression of *Edg2* in the cortex may contribute to the loss, or reduction in function, of interneurons underlying the network dysfunction seen in schizophrenia (section 1.1.4).

qRT-PCR data showing increased *Edg2* expression in the prefrontal cortex of rats chronically treated with PCP and in post-mortem dorso-lateral prefrontal cortex of schizophrenic patients were consistent with this hypothesis, suggesting that the role of Edg2 in cortical regions was indeed critical in schizophrenia, and interestingly, was somehow related to altered NMDA receptor transmission. Further support to this hypothesis was provided by *in situ* hybridisation data showing an increased expression of *Edg2* in the prelimbic and infralimbic cortices of rats having undergone a behavioural task of executive function (section 3.5.4), suggesting that

neuronal *Edg2* was more important for schizophrenia-related cognitive tasks than white matter *Edg2*.

Further pathway analysis of gene expression data obtained from the microarray performed in the rat chronic PCP model and from a microarray investigating gene expression in the post-mortem dorso-lateral prefrontal cortex of schizophrenic patients (Hiromitsu Ozeki) allowed the development of a hypothesis about the mechanism whereby Edg2 may contribute to schizophrenia pathophysiology and how this may be related to NMDA receptor function. Thus, Edg2 was suggested to couple to a common signalling cascade with NMDA receptors, leading from Ptk2b (Pyk2) to the MAP kinase Mapk9 (Jnk2) and held together by adaptor proteins such as Nck1 -hence the proposed "Pyk/Nck" pathway. Importantly, potential neurotoxicity of the NMDA receptor antagonist PCP has to be excluded as the mechanism inducing decreased activity within this cascade since the dose treatment regime used in the chronic PCP model (Cochran *et al.* 2003) was specially developed to ensure that neuronal loss could not account for the neurochemical and behavioural deficits of the model. Conversely, further support for the involvement of this cascade in schizophrenia may interestingly be provided by a number of interactions, suggested in the literature, between members of the "Pyk/Nck" pathway and other pathways or mechanisms potentially implicated in schizophrenia. These include in particular the role of Mapk9 in the activation of gene transcription (Ham *et al.* 2000) and the role of the Nck1-interacting protein Pak1 in synaptic structure, in the regulation of glutamate receptor levels and potentially in the modulation of gene transcription and synaptic plasticity through indirect interaction with the calcium/calmodulin-dependent protein kinase IV (CamKIV) (Kang *et al.* 2001).

The hypothesis that restoring activity within the "Pyk/Nck" cascade may constitute a route to counterbalance PCP-induced deficits in the rat chronic PCP model and NMDA receptor hypofunction in schizophrenic patients was confirmed *in vitro* using a stable cell line overexpressing *EDG2* receptors: in these cells, EDG2 activation was shown to induce gene expression changes within the proposed "Pyk/Nck" cascade. In addition, despite the limited specificity of available antibodies against MAP kinases, consistent evidence was obtained from western blots and ELISA experiments which strongly suggested that EDG2 activation in these cells induced increased phosphorylation -hence activation- of Jnk2, the final output of the "Pyk/Nck" pathway. Nevertheless, further studies would be necessary to maintain this

assumption, including use of EDG2 agonist compounds and more specific investigation of the activation of the "Pyk/Nck" pathway, both *in vitro* (using different cell lines as well as primary neurons) and *in vivo*. Moreover, although this evidence suggested that Edg2 activation may provide a way to counterbalance potential deficits within the "Pyk/Nck" pathway, use of EDG2 agonist compounds in NMDA receptor hypofunction-related contexts would be necessary to assess the differential up- and downregulation of the "Pyk/Nck" pathway signalling activity *in vitro* and *in vivo* after induction of NMDA receptor hypofunction by PCP and after stimulation of EDG2 receptors by specific agonists. In particular, such evidence would establish the pharmacological proof of concept that is required to further support the development of EDG2 agonists as compounds acting at the "Pyk/Nck" cascade and thereby as promising compounds for the treatment of schizophrenia.

Despite this lack of irrefutable evidence that Edg2 stimulation may counterbalance NMDA receptor hypofunction in schizophrenia, literature supporting the involvement of *Edg2* in schizophrenia stimulated the use of EDG2 agonists, developed in other respects (Santos *et al.* 2004), in the chronic PCP model of schizophrenia and the investigation of critical deficits particularly relevant to some aspects of schizophrenia lacking effective therapeutics. *In vivo* experiments using EDG2 agonists were thus undertaken in the laboratory (Susan Cochran, Alice Egerton, Allan McVie) and *in vivo* evidence of the potential of EDG2 compounds in the treatment of schizophrenia was in fact obtained prior to any conclusive *in vitro* evidence, with data showing EDG2 agonist-induced reversal of PCP-induced sensorimotor gating deficits in prepulse inhibition (PPI) (Alice Egerton) and deficits in prefrontal cortex metabolic hypofunction (Susan Cochran and Allan McVie). The latter results may appear particularly promising since this ability of EDG2 compounds may demonstrate an improved efficacy of these compounds compared to clozapine, one of the best atypical antipsychotic drugs, commonly used as a reference in pharmacological studies attempting at developing better treatments for schizophrenia, and which does not have the ability to reverse the critical deficits in prefrontal cortex metabolic hypofunction induced by chronic PCP treatment (Cochran *et al.* 2003).

Whether this activity of EDG2 agonists occurs through the activation of the "Pyk/Nck" pathway still needs to be determined. However, concomitant evidence of

the ability of EDG2 stimulation to trigger the activation of the NMDA receptor-downstream "Pyk/Nck" cascade *in vitro* and to reverse critical deficits in the chronic PCP model of schizophrenia *in vivo* provided further support to the hypothesis that this cascade may represent a core molecular basis of the chronic PCP model which may reflect of a critically dysfunctional pathway in schizophrenia in humans.

In vitro and *in vivo* evidence providing further evidence that EDG2 may potentially represent a promising drug target for schizophrenia stimulated the development of a an assay to be used for screening potential EDG2 agonists. The [^{35}S]-GTPγS binding assay optimised for use with the stable transformed *EDG2*-overexpressing cell line was thus confirmed to reliably detect dose-dependent activation of EDG2 receptors (and potential GPR23 receptors expressed in these cells) by LPA. Although use of specific EDG2 agonists and antagonists would be necessary to definitely conclude on its specificity and sensitivity, the ability of this [^{35}S]-GTPγS binding assay, shown for LPA, to allow the calculation of exact pharmacological parameters, supported its use and its potential value in screening the agonist properties of potential EDG2 compounds.

Generation of the hypothesis that EDG2 receptors were coupled to a cascade dysfunctional in the chronic PCP model and in schizophrenic patients so that their activation may constitute a way to counterbalance deficits critical to schizophrenia pathophysiology, was first driven by concomitant evidence both obtained through YRING microarray results (*Edg2* and *EDG2* both showing increased expression in the prefrontal cortex of rats chronically treated with PCP and in post-mortem dorso-lateral prefrontal cortex of schizophrenic patients respectively) and from the literature about *Edg2*, showing that *Edg2* knock-out mice displayed behavioural deficits and neurochemical changes similar to those observed in schizophrenia. In other terms, the chronic PCP model and *Edg2* knock-out mice, whih both show schizophrenia-like behaviour, showed opposite directions in *Edg2* expression changes. These concomitant but opposite changes provided therefore strong support of the involvement of this gene in schizophrenia pathophysiology, but also suggested that in one of these animals, the changes in expression were likely to reflect a compensatory mechanism whereas in the other they may be causative. Interestingly, the observed changes in the expression of *Edg2* in the chronic PCP model were

proposed to be related to expression changes of a number of genes with which Edg2 may interact as part of a signalling cascade located downstream of NMDA receptors. The work presented in this thesis together with additional data obtained in the lab provided strong evidence in support to this hypothesis.

Nevertheless, the core foundations of this hypothesis of *Edg2* overexpression being a compensatory mechanism attempting to restore activity within the "Pyk/Nck" pathway may well be doubted since this theory does not take into account the expression of *Edg2* in glial cells, which is so predominant that it was long thought to be its exclusive cellular localisation. Thus, although the levels of expression do not necessarily reflect biological significance, it may be suggested that it is this loss of glial *Edg2* that is responsible of the schizophrenia-like phenotype of *Edg2* knock-out mice, rather their lack of low neuronal *Edg2* expression. In the light of the potential role of Edg2 in myelination, this hypothesis may be consistent with the concept that schizophrenia may be primarily a disorder of neurodevelopment, in which dysmyelination may account for critical alterations in brain and cortical connectivity in schizophrenia.

Following on this theory, hypotheses may have to be proposed to explain the changes in *Edg2* expression observed in the rat chronic PCP model and those of *EDG2* in schizophrenic patients, assuming that these changes were genuine and relevant to schizophrenia. Depending on whether these detected changes reflected increased glial or neuronal expression of *Edg2*, several hypotheses may be proposed (including the hypothesis that increased neuronal *Edg2* activation may induce a retraction of neurites thereby altering synaptic architecture and connectivity). However, how these changes may result from PCP-induced NMDA receptor hypofunction may be difficult to explain.

Conversely, evidence showing that NMDA receptor antagonism and loss of Edg2 function induced a range of similar deficits, including not only behavioural impairments and neurochemical changes but also comparable alterations in entorhinal gamma oscillations (which constitute markers of interneuronal function and interneuron-mediated brain rhythms and are associated with cognitive function) (Cunningham *et al.* 2006), recently provided further support to the hypothesis that

increased neuronal expression of *Edg2* in the prefrontal cortex constituted indeed an attempt at restoring NMDA receptor hypofunction in PCP-treated rats and in schizophrenic patients.

In conclusion, these results provided very strong evidence that stimulation of EDG2 receptors may represent a very promising approach for the treatment of schizophrenia and may lead to the development of compounds of improved efficacy towards the negative symptoms and the cognitive deficits that currently lack effective therapeutics. Moreover, the "Pyk/Nck" pathway identified in this work may well represent a core mechanism of schizophrenia since it may provide a link not only between NMDA receptor hypofunction and EDG2 but also with a number of mechanisms underlying alterations in synaptic structure, glutamate receptor levels and synaptic plasticity-associated gene transcription in schizophrenia. Interestingly, further investigation of the activation of the "Pyk/Nck" pathway and of the mechanisms whereby EDG2 agonists exert their effect may benefit from the availability of such compounds, developed in the context of other diseases. Overall, EDG2 receptors have indeed emerged as a promising therapeutic target for several diseases including dysmyelinating disorders (Beer *et al.* 2000) but also, beyond neurobiology, for a range of cancers, particularly colorectal cancers for which EDG2 was proposed to represent a very potential chemopreventive target (Shida *et al.* 2004).

6.3- *KIAA1189*, a novel gene for schizophrenia?

Following on the same innovative strategy of attempting to identify novel schizophrenia-associated genes and first reflected by the use of the partially EST-based Affymetrix RG-U34 GeneChips, the EST AI072720 was selected as a candidate gene for which to investigate and characterise in relation with schizophrenia. Initial support for the genuine expression of this EST in the rat brain and its potential relevance to schizophrenia (provided by qRT-PCR confirmation of the differential expression of the EST AI072720 in the prefrontal cortex of the chronic PCP model of schizophrenia) did indeed stimulate further analysis of this

EST, potential identification of a transcript that it may be related to and characterisation of this transcript in relation with schizophrenia. Molecular biology techniques were used for this aim and 5' Rapid Amplification of cDNA Ends (5'RACE) PCR provided confirmation that the EST AI072720 was genuinely expressed as part of a rat predicted gene called *RGD1308367*, which had been suggested by the bioinformatics analysis of its surrounding genomic sequence (section 5.2). Interestingly, although "*similar to KIAA1189 protein*" (*i.e.* the description of rat *RGD1308367* gene) was mostly uncharacterised, the putative protein was predicted to have a C-terminal ezrin-radixin-moesin (ERM) actin-binding domain suggestive of a potential role in cytoskeletal architecture which further reinforced the interest in studying this gene in relation with schizophrenia.

Importantly, identification of *RGD1308367* as a rat transcript, yet uncharacterised but predicted, for EST AI072720 paved the way for further characterisation studies not only of the rat gene but also of its human orthologue, *KIAA1189*. In particular, this allowed the investigation of *KIAA1189* expression in post-mortem tissue from schizophrenic patients and genotyping of single nucleotide polymorphisms (SNPs) within *KIAA1189* in blood samples from schizophrenic patients, of which both sample types were available in the laboratory. Very interestingly, using qRT-PCR, *KIAA1189* expression was shown to be differentially increased in post-mortem dorso-lateral prefrontal cortex samples from the Harvard and Brain-Net collections of schizophrenic patients, consistent with the increased expression of *RGD1308367* confirmed by qRT-PCR in the prefrontal cortex of rats treated with PCP according to the chronic PCP model treatment regime (Cochran *et al.* 2003). Moreover, although genotyping of three SNPs within the human *KIAA1189* gene did not provide evidence of genetic association between *KIAA1189* and schizophrenia in a case-control population of 600 schizophrenic patients and controls, this probably did not constitute sufficient data to draw any conclusion since it is likely that the design of this experiment was not sophisticated enough to yield any positive finding. As discussed in section 5.12.3, insufficient SNP density, localisation of the SNPs examined in different haplotype blocks and heterogeneity on the collection of populations are among all the issues that may explain the negative results. Compared to the most recent association studies performed in schizophrenia, such as that of Gurling *et al.* (2006) investigating the effect of a genetic susceptibility on an

endophenotype in carefully selected populations of schizophrenic patients (Gurling *et al.* 2006), the design of this SNP association study may appear too simplistic, suggesting that further experiments including more complex designs and analyses may be needed to yield positive findings and to further explore this aspect of the characterisation of *KIAA1189* in schizophrenia.

Finally, the genomic localisation of *KIAA1189* at chromosome 2q is a key schizophrenia locus pointed out in several linkage studies and recently associated with visual working memory. Thus, this evidence interestingly added to *RGD1308367* and *KIAA1189* expression results, providing further support to the hypothesis that *KIAA1189* may be an intriguing novel candidate gene for schizophrenia.

In order to provide some insight on the potential function of *RGD1308367*, *in situ* hybridisation was performed using probes designed within its predicted sequence, as close as possible to the location of the probes used in *RGD1308367* qRT-PCR. *RGD1308367* was shown to exhibit a specific expression pattern, which was, again, consistent with that of EST AI072720, providing evidence that the EST AI072720 and *RGD1308367* were likely to be the same transcript, the EST AI072720 being part of the 3' untranslated region of *RGD1308367*. Very high expression of *RGD13083637* within all major white matter tracts and much lower expression within all cortical and sub-cortical areas suggested that *RGD1308367* was predominantly expressed in glial cells. Moreover, although *in situ* hybridisation analysis of EST AI072720 and *RGD1308367* expression did not provide consistent semi-quantitative results, *RGD1308367* expression was found to be decreased in the fornix and the corpus callosum of rats treated with PCP according to the chronic PCP treatment regime by comparison with rats treated with a vehicle (saline). Interestingly, because these white matter structures are essential to the connectivity between brain structures and cerebral hemispheres (respectively), changes in gene expression, hence functional impairment, within such regions may be consistent with the hypothesis of schizophrenia being a disorder of cortical connectivity (section 1.1.5.1). Interestingly, concomitant to writing up this work, a paper was published describing the characterisation of *KIAA1189*, hereby called Ermin, as a myelinating oligodendrocyte-specific protein (Brockschnieder *et al.* 2006). To some

extent, the data presented in this paper may be considered further support for the hypothesis that changes in glial *RGD1308367* expression may be related to alterations in the myelination process which is critical to proper connectivity within the brain.

However, although both hypotheses do not have to be exclusive, evidence showing increased expression of *RGD1308367* and its human *KIAA1189* orthologue in the chronic PCP model and in schizophrenia patients, respectively, was strongly evocative of another potential mechanism as to the involvement of *RGD1308367/KIAA1189* in schizophrenia. Data showing *RGD1308367* expression in laser-capture microdissected prelimbic cortex (Antonio Ferra and Catherine Winchester) provided further support for this hypothesis by confirming that *RGD1308367* was not only expressed in white matter but also in a region of the brain densely populated with neurons.

Following on both hypotheses, KIAA1189 was overexpressed as a fusion protein with an N-terminal FLAG epitope not only in glial- but also in neuronal-like cells, to investigate its subcellular localisation. In either rat C6 glioma or differentiated PC12 cells, KIAA1189 overexpression did not induce any apparent phenotype and very similar subcellular localisation of KIAA1189 was shown in both cell lines, not providing any insight into the potential role. Nevertheless, its subcellular localisation in the plasma membrane and within neurites was consistent with the potential actin-binding role of the predicted C-terminal ERM domain of KIAA1189 and with expression of typical ERM proteins at the cytoplasmic surface of membranes (Sato *et al.* 1991). Moreover, although *in situ* hybridisation did not have the power to precisely identify the subcellular localisation of the mRNA, consistency between the expression of KIAA1189 in neurites and the putative expression of *RGD1308367* mRNA, suggested from *in situ* hybridisation data, within the axons of cortical neurons suggested that KIAA1189 may be, at least partially, locally translated within the axons and may thus play a critical role in the regulation of synaptic plasticity (Piper and Holt 2004).

Although immunoprecipitation studies did not demonstrate interaction between KIAA1189 and actin, maybe due to technical issues (including the possibility that the N-terminal FLAG® epitope may alter KIAA1189 folding, that the protein may need to be activated or that the cell line used in these experiments may not be suitable),

KIAA1189 was shown to colocalise with actin in some processes of differentiated PC12 cells, at the end of some neurite tips and in the plasma membrane, suggesting that it may have a critical role at cell membrane-actin adhesion sites and in the architecture and extension of neurites.

Very interestingly, such a role of KIAA1189 in neurite outgrowth was confirmed by overexpressing KIAA1189 in non-differentiated PC12 cells and examining neurite outgrowth in the course of NGF-induced differentiation, both by semi-quantitatively measuring neurite length and by looking at epifluorescence of a co-transfected GFP protein. Both methodologies gave consistent and reliable results, showing reduced neurite length in KIAA1189-overexpressing cells by comparison with cells transfected with the empty FLAG expression vector.

That this phenotype of KIAA1189-overexpressing cells had not been detected in initial experiments attempting to determine the subcellular localisation of KIAA1189 may not be surprising due to the essential difference between both studies in terms of timescale of KIAA1189 transfection and NGF-induced differentiation. The FLAG-KIAA1189 fusion protein was indeed initially overexpressed in differentiated PC12 cells while in the neurite outgrowth assay, PC12 cells were first transfected prior to being treated with NGF to induce the differentiation process. Therefore, it may be suggested that KIAA1189 has a critical role during the process of differentiation rather than in differentiated cells, which may explain failure to confirm KIAA1189 interaction with actin and to show any phenotype in the initial studies.

Extrapolating *in vitro* evidence into *in vivo* hypotheses always requires extreme caution but even more when attempting to relate *in vitro* results (obtained using a particular cell type etc) to complex disease mechanisms such as those occurring in schizophrenia, which involve disturbances within networks rather than single cells or specific cell types. Nevertheless, evidence that KIAA1189 overexpression may affect neurite extension, together with gene expression results showing an increased expression of *RGD1308367/KIAA1189* in the rat chronic PCP model and in schizophrenic patients, not only further confirmed that KIAA1189 was involved in schizophrenia but strongly suggested that the mechanisms whereby it may play a role in schizophrenia were likely to be somehow related to alterations in the processes of the formation of cortical networks during the development of the brain. Probably by interacting with actin at critical times of development, KIAA1189 probably plays a

critical role in the basic steps of brain histogenesis (cell production, migration, neurite outgrowth and formation of the synaptic circuitry) so that subtle alterations in its expression may lead to abnormalities in neuronal connectivity contributing to schizophrenia pathogenesis (Sawa and Snyder 2002).

However, this hypothesis of the critical role of KIAA1189 during development as potentially contributing to susceptibility to schizophrenia cannot be valid as such in the context of a pharmacological model such as the chronic PCP model, in which it is the chronic administration of PCP to adult animals that somehow induced changes in its (or exactly in that of its rat orthologue *RGD1308367*) expression. In this context, evidence of an axonal translation of *KIAA1189*, based on *in situ* hybridisation results and *in vitro* subcellular localisation studies using overexpressed proteins, may provide the foundations of another mechanism whereby KIAA1189 may play a role in relation to schizophrenia. Axonal translation has indeed been shown to play a critical role in providing a flexible and efficient way of modulating synaptic plasticity and long-term potentiation (LTP, *i.e.* the strengthening of synapses which occurs in response to learning and experience), both processes requiring synaptic activation of NMDA receptors. Thus, chronic administration of the NMDA receptor antagonist PCP necessarily affects this process, reflected at the molecular level by dramatic structural changes of dendritic spines associated with actin polymerisation. Although the extent to which spines remain plastic in the adult brain is unclear, this hypothesis may also be true at mature synapses where a process similar to LTP was shown whereby NMDA receptor activation triggers spine expansion and actin polymerisation (Lang *et al.* 2004). Thus, the expression of KIAA1189 in neuronal-like processes (where it may be locally translated) of mature neurons may suggest a role for KIAA1189 in the actin dynamics-related structural changes of dendritic spines induced by decreased NMDA receptor activity. Whether the changes in *KIAA1189* expression in the chronic PCP model actually constitute an attempt at counterbalancing the alteration of synaptic plasticity induced by PCP-mediated decreased NMDA receptor activity or reflect its contribution to further altering these processes remains unknown. Further experiments such as additional immunofluorescence studies, use of actin dynamics-perturbing agents and histological examination of dendritic spines would be necessary to refine this hypothesis and provide further insight into these complex mechanisms.

In conclusion, although all these findings may be considered preliminary since most of, if not all, experiments would benefit from improved designs including increasing sample size etc, they provide very intriguing genetic, genomic and functional evidence suggesting that KIAA1189 may have role in schizophrenia susceptibility and/or physiopathology. Probably the most important caveats of this work lay in the functional part of KIAA1189 characterisation, with the fact that the techniques used 1) did not have the power to determine the cellular localisation of RGD1308367 protein, *i.e.* if RGD1308367 was expressed in neurons and/or in glial cells (and in which cell type etc) and to what extent; and 2) were limited, due to the unavailability of an antibody against RGD1308367/KIAA1189, to the functional characterisation of exogenous proteins, which may disturb normal cellular physiology and may not exactly reflect the function of endogenous proteins.

Nevertheless, although for these reasons no precise mechanism as to the role of KIAA1189 in schizophrenia can be proposed on the basis of this work, we suggest as a general hypothesis that KIAA1189, probably by directly or indirectly interacting with actin, plays a role in the extension of neurites which occurs during the development of the brain and to some extent underlies the formation of cortical networks critical for brain connectivity, and is involved in the modulation of synaptic plasticity in adults by mediating NMDA receptor-dependent dendritic spine changes.

In addition, expression of KIAA1189 in oligodendrocytes, shown by (Brockschnieder *et al.* 2006), may suggest a role for KIAA1189 in myelination.

Whatever the mechanism, all these hypotheses may be consistent with the current hypotheses suggesting that schizophrenia is a neurodevelopmental disorder in which alterations in synaptic plasticity and connectivity probably represent the core molecular foundations that underlie the complexity of the schizophrenia phenotype. Therefore, *KIAA1189* may represent a very intriguing novel candidate gene for schizophrenia.

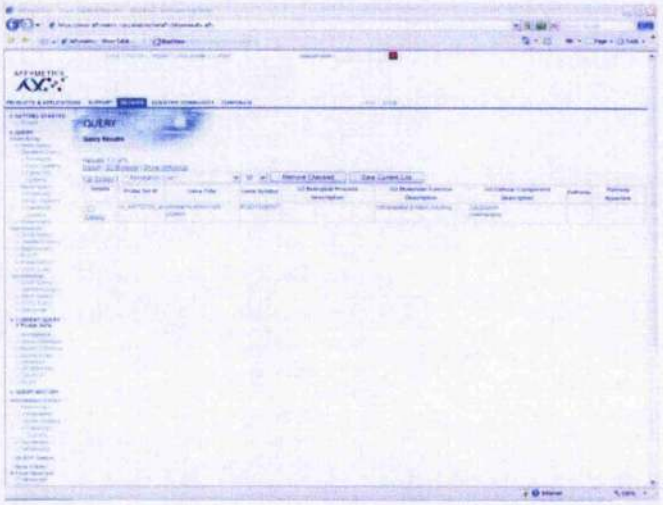
6.4- Final conclusion

The data presented in this thesis represent very promising findings for schizophrenia which may help, in the short or longer term, in the development of novel antipsychotic drugs. Through the identification of two novel schizophrenia-associated genes using one of the best validated models of schizophrenia, the rat chronic PCP model, and through the combination of different approaches that is necessary to lay the foundations of any hypothesis about a such complex disorder, they provide a very valuable insight into the mechanisms potentially involved in the pathophysiology of schizophrenia and more particularly into the mechanisms underlying the critical negative and cognitive deficits which currently lack effective therapeutics. In this respect, evidence suggesting that EDG2 agonists may have an atypical antipsychotic activity makes EDG2 a particularly promising novel drug target for schizophrenia. In addition, although further studies are needed to refine the potential role of KIAA1189 in schizophrenia, this previously completely uncharacterised gene appears very promising, especially since its role may allow the bridging of all major current hypotheses of schizophrenia.

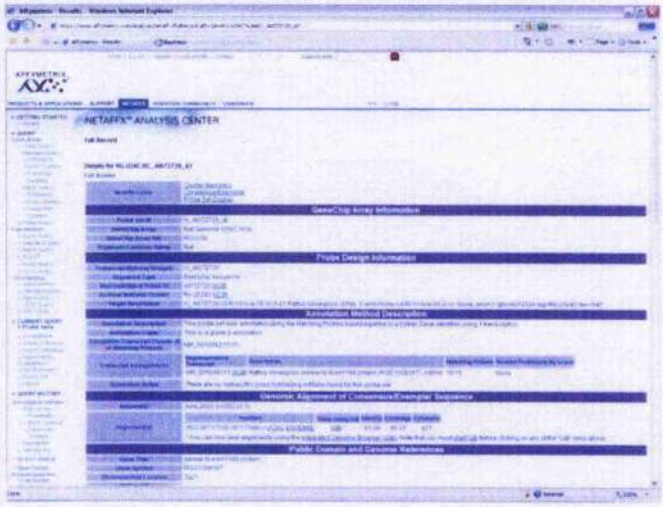
- APPENDICES -

Appendix A.

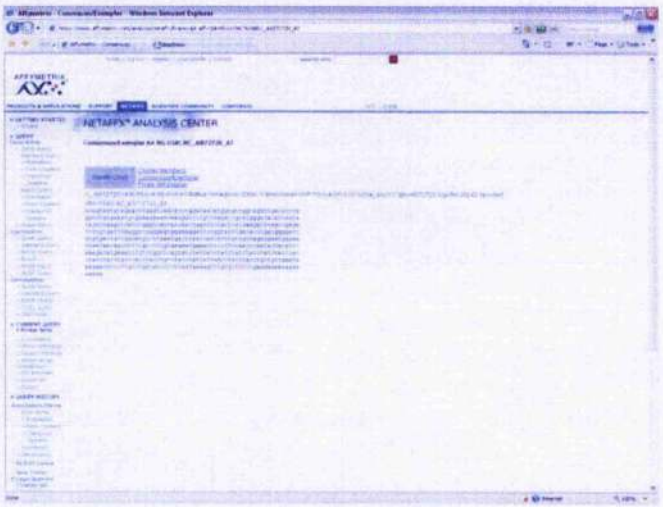
Screenshots of the different stages of the bioinformatics analysis process



Screen displaying probe retrieval at NetAffx™ website

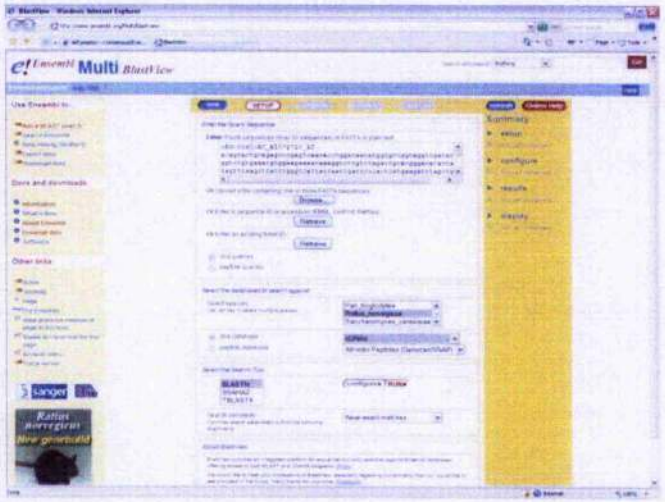


Screen displaying probe set information at NetAffx™ website



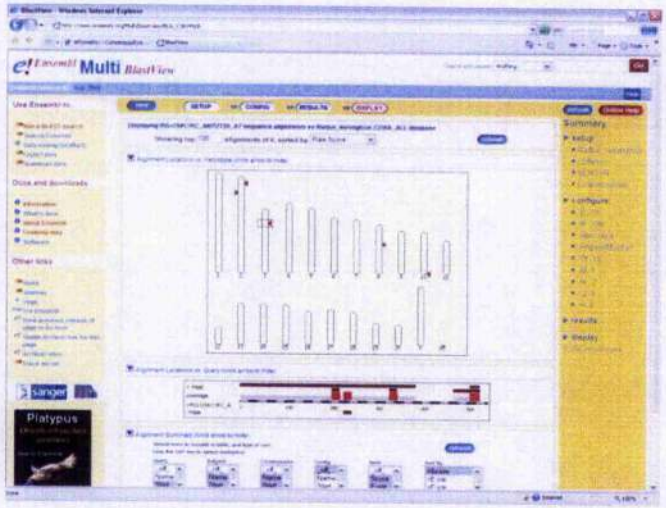
Probe set sequence retrieval at NetAffx™ website

From here on, the bioinformatics homology screening process is shown for one species only (rat), it was then repeated to align each retrieved probe set against mouse and human databases. The following manual analysis of these alignments is shown in figure 3.1 A-C, which represent examples of homology screens allowing good, uncertain and unsuccessful conversion into rat genes.

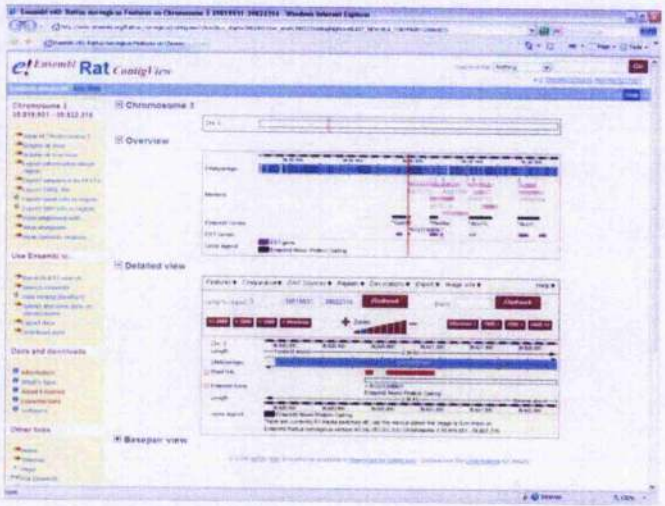


ENSEMBL Blast entry screen: probe set sequence copied from NetAffx™ was entered to be aligned against rat cDNA database

ENSEMBL Blast processing screen



ENSEMBL screen displaying a genome overview of alignments of entry sequence



ENSEMBL screen displaying a view of the contig where the entry sequence showed the best alignment

ENSEMBL screen displaying information about the gene potentially identified from aligning the probe sequence against rat cDNA database

Appendix B.

Homology screening results, 66 identified genes

CONFIDENT	Affy description	Chip	% fold change (if >20%)	Rat gene	Human gene	Human locus		Function	Previous association with schizophrenia ?	Classification	Potential interest for validation
rc_AI011891_at	Rattus norvegicus transcribed sequences	RGU34 B		Novel gene (antisense)	NM_015113 (cDNA and gen antisense)	17p13.2 (minus strand)	No description	unknown	No	antisense to the known gene	
rc_AI059356_at	No description	RGU34 B		A2S3_RAT (antisense)	ALS2CR3	2q33.1	Amyotrophic lateral sclerosis 2 chromosomal region candidate gene protein 3	"gene ontology : receptor binding, intracellular transporter activity, neurotransmitter transport, cytoplasm, kinesin complex, plasma membrane ; tissue specificity : widely expressd, with highest expression in heart	Yes in theory, but not worth taking into account (Hahimoto Thyroiditis)	antisense to the known gene	
rc_AA945803_g_at	Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_009206.1 (H.sapiens) lung cancer candidate [Homo sapiens]	RGU34 B		Novel gene (antisense)	PDAP2	3p21.31 (minus strand)	FUS-1 protein (Fusion 1 protein)	May function as a tumor suppressor, inhibiting colony formation, causing G1 arrest and ultimately inducing apoptosis in homozygous 3p21.3 120-kb region-deficient cells. Belongs to the Fus1 family	No	antisense to the known gene	
rc_AA997765_at	fibrillin-1	RGU34 B		NM_031825 (consensus matches about 600bp away from the gene)	FBN1 (gen seq about 600bp away from FBN1 gene)	15q21.1	Fibrillin 1 Precursor	Structural component of connective tissue microfibrils that binds calcium. Fibrillin-1 containing microfibrils provide long-term force bearing structural support	15q15 (SCZD10)	Cytoskeletal architecture	Yes
rc_AI009450_at	Rattus norvegicus transcribed sequence with strong similarity to protein pir:A37098 (H.sapiens) A37098 gelation	RGU34 B		Novel gene	FLNA	Xq28	Filamin A (Alpha-filamin, filamin 1, endothelial actin-binding protein ABP-280, nonmuscle filamin)	Peripheral cytoplasm. Promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins. Anchors various transmembrane	No	Cytoskeletal architecture	

	factor ABP-280, long form - human							proteins to the actin cytoskeleton and serves as a scaffold for a wide range of cytoplasmic signaling proteins			
rc_AA997630_at	"Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_002304.1 (H.sapiens) actin-binding LIM protein 1, isoform a; limatin; actin-binding LIM protein; actin binding LIM protein 1; abLIM; LIM actin-binding protein-1; LIM actin binding protein 1 [Homo sapiens]"	RGU34 B		Novel gene	ABLIM1	10q25.3	Actin-binding LIM protein 1 isoform A, Lim actin-binding protein 1, limatin, actin-binding Lim protein	gene ontology : actin binding, electron transporter activity, electron transport, cytoskeleton organization and biogenesis, histogenesis and organogenesis, vision, actin cytoskeleton, actin binding, cytoskeleton organization and biogenesis	No	Cytoskeletal architecture	
rc_AI058595_at	mucin and cadherin-like	RGU34 B		NM_138525	MUCDHL (cDNA antisense, gen sense)	11p15.5	Mu-Protocadherin isoform 3, mucin and cadherin-like protein, MUCDHL-FL, MUCDHL-ALT	gene ontology : calcium ion binding, homophilic cell adhesion, membrane	No, but coincides with DRD4 locus	Cytoskeletal architecture (synapse)	Yes
rc_AA955151_at	hypothetical protein RMT-7	RGU34 B		NM_145084	NM_017750	2p11.2	No description, Protein family:ambiguous	gene ontology : electron transport	No	Electron transport	
rc_AI012785_at	Rattus norvegicus transcribed sequence with weak similarity to protein ref:NP_061898.1 (H.sapiens) chromosome 20 open reading frame 16 [Homo sapiens]	RGU34 B		Novel gene	NM_152911	10q26.3	Peroxisomal N1-acetyl-spermine/spermidine oxidase	gene ontology : electron transport	No	Electron transport	
rc_AI073006_s_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_115635.1	RGU34 C		Novel gene	NM_032259	16p13.3	No description, protein family: ambiguous	gene ontology : electron transporter activity, electron transport	No	Electron transport	

	(H.sapiens) hypothetical protein DKFZp434P054 [Homo sapiens]										
rc_AI060282_at	perosisomal 2-enoyl-CoA reductase	RGU34 B		NM_133399	NM_018441	2q35	Peroxisomal trans 2-enoyl coA reductase, putative short chain alcohol dehydrogenase	gene ontology : apoptosis regulator activity, oxidoreductase activity, apoptosis, metabolism	No	Metabolism	
rc_AI059022_at	Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_054759.1 (H.sapiens) PTD015 protein [Homo sapiens]	RGU34 B		Novel gene (100% over 88bp !)	NM_014040	11q13.5	PTD015 protein	gene ontology : sugar porter activity, transport, phosphoenolpyruvate-dependent sugar phosphotransferase system	No, but 11q14-q21 : SCZD2	Metabolism	
rc_AI029499_at	"Rattus norvegicus transcribed sequence with weak similarity to protein ref:NP_072090.1 (H.sapiens) aldehyde dehyrdogenase 8 family, member A1; aldehyde dehydrogenase 12; aldehyde dehydrogenase 8 family, member A1 [Homo sapiens]"	RGU34 B		Novel gene	NM_153329	19q13.33	No description, protein family:aldehyde dehydrogenase	gene ontology : oxidoreductase activity, metabolism	No (APOE on locus 19q13.2)	Metabolism	
rc_AI011300_at	"Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_062823.1 (H.sapiens) solute carrier family 7, member 10; asc-type amino acid transporter 1; solute carrier family 7, (cationic	RGU34 B		AAA1_MOUSE	SLC7A10	19q13.11	ASC-type amino acid transporter 1 (ASC-1)	Integral membrane protein (probable). Sodium independent, high affinity transport of small neutral D- and L-amino acids. May play a role in the modulation of glutamatergic transmission through mobilization of D-serine at the glutamatergic synapse	No (APOE on locus 19q13.2)	Metabolism (role in glutamatergic transmission ?)	Yes

	amino acid transporter, y+ system) member 10 [Homo sapiens]"										
rc_AA818692_at	"Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_004882.1 (H.sapiens) mitochondrial ribosomal protein L33; ribosomal protein L33-like; chromosome 2 open reading frame 1 [Homo sapiens]"	RGU34 C		Novel gene (sense)	MRPL33 (cDNA antisense, gen sense)	2p23.2 (plus strand)	Mitochondrial ribosomal protein L33 isoform A, ribosomal protein L33-like, chromosome 2 open reading frame 1	gene ontology : structural constituent of ribosome, protein biosynthesis, intracellular, ribosome	No	Mitochondrial	
rc_AI008223_at	ATPase inhibitor	RGU34 B	22	IATP_RAT	ATPIF1 (cDNA antisense, gen sense)	1p35.3	ATPase inhibitor, mitochondrial precursor	Mitochondrial. Thought to be a regulatory component of the ATP-synthesizing complex in the mitochondria (by similarity). Belongs to the ATPase inhibitor family	No	Mitochondrial	
rc_AI170002_at	Rattus norvegicus Aa1027 mRNA, complete cds	RGU34 C		ENSRNOG0000000 5203 (Novel gene)	SC1G_HUMAN	7p11.2	Protein transport protein SEC61 Gamma subunit	Tail-anchored membrane protein. Necessary for protein translocation in the endoplasmic reticulum. Heterotrimeric complex composed of SEC61-alpha, SEC61-beta and SEC61-gamma. Belongs to the SEC61/SEC61-gamma family.	No	Protein transport	
rc_AI137761_at	Rattus norvegicus transcribed sequence with strong similarity to protein sp:Q9UGP8 (H.sapiens) SC63_HUMAN Translocation protein SEC63 homolog	RGU34 C		None detected (cDNA) Rat EST matches with gen seq about 750bp away from novel gene (human SEC63 homolog)	SEC63	6q21 (minus strand)	Translocation protein SEC63 homolog	Integral membrane protein. Endoplasmic reticulum. Required for preprotein translocation. Complex that contains SEC61, SEC62 and SEC63. Contains 1 J domain	Yes (6q21-25 : cited by O'Donovan et al, Hum Mol Genet, 2003)	Protein transport	Yes ?
rc_AI059504_s_at	parathyroid	RGU34		PTRR_RAT	PTHR1	3p21.31	Parathyroid	Integral membrane	No	Receptor	

	hormone receptor 1	B					hormone/Parathyroid hormone-related peptide receptor precursor (PTH/PTHR receptor)	protein. Receptor for parathyroid hormone and for parathyroid hormone-related peptide. Activity mediated by G proteins which activate adenyl cyclase and also a phosphatidylinositol-calcium second messenger system			
rc_AI058759_at	Rattus norvegicus transcribed sequences	RGU34 B		Novel gene	EPHA2	1p36.13	Ephrin type-A receptor 2 precursor (EC 2.7.1.112, tyrosine-protein kinase receptor ECK, epithelial cell kinase)	Type I membrane protein. Receptor for members of the ephrin-A family. Binds to ephrin-A1, -A3, -A4 and -A5	No	Receptor	
rc_AI171162_at	Rattus norvegicus transcribed sequence with moderate similarity to protein pir:A35329 (H.sapiens) A35329 EVI2 protein precursor - human	RGU34 C	26	None detected	EVI2A	17q11.2	EVI2A protein precursor (ecotropic viral intergration site 2A protein)	Type I membrane protein. May complex with itself or/and other proteins within the membrane, to function as part of a cell-surface receptor	No	Receptor	Yes
rc_AI112149_at	Rattus norvegicus transcribed sequences	RGU34 C	29	EDG2_MOUSE	EDG2	9q31.3	Lysophosphatidic acid receptor EDG-2 (LPA receptor 1, LPA-1)	Integral membrane protein. Receptor for lysophosphatidic acid (LPA), a mediator of diverse cellular activities. Seems to be coupled to the G(i)/G(0), G(12)/G(13), and G(q) families of heteromeric G proteins. Belongs to family 1 of G-protein coupled receptors	No	Receptor	Yes
rc_AI010050_at	"Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_004805.1 (H.sapiens) U5 snRNP-specific 40 kDa protein (hPrp8-binding), prp8, U5 snRNP-	RGU34 B		Novel gene (antisense)	NM_004814 (cDNA and gen antisense)	1p35.2 (minus strand)	U5 SNRNP-specific 40 KDa protein (HPRP8-BINDING, PRP8, U5 SNRNP-specific 40 Kda protein)	gene ontology : pre-mRNA splicing factor activity, mRNA splicing, RNA processing, nucleus, snRNP U5, small nucleolar ribonucleoprotein complex	No	RNA-binding	

	specific 40 kDa protein [Homo sapiens]"										
rc_AI136838_at	endothelial monocyte activating polypeptide 2	RGU34C		Novel gene	SCYE1	4q24	Multisynthetase complex auxiliary component P43 (contains: endothelial-monocyte activating polypeptide II (EMAP-II)) (small inducible cytokine subfamily E member 1)	"gene ontology : tRNA binding, cytokine activity, amino acid activation, chemotaxis, inflammatory response, signal transduction, cell-cell signalling, extracellular space ; contains 1 tRNA-binding domain"	No (EGF on locus 4q25)	RNA-binding	
rc_AA818026_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_003745.1 (H.sapiens) eukaryotic translation initiation factor 3, subunit 5 (epsilon, 47kD) [Homo sapiens]	RGU34C		Novel gene (sense)	EIF3S5	11p15.4	Eukaryotic translation initiation factor 3 subunit 5 (eIF-3 epsilon, EIF3 p47 subunit, EIF3f)	Binds to the 40S ribosome and promotes the binding of methionyl-tRNAi and mRNA. Associates with the complex P170-EIF3. Belongs to the PSMD7/C6.1A family	No (DRD4 on locus 11p15.5)	RNA-binding	
rc_AI071773_at	"Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_075605.1 (H.sapiens) one twenty two protein; hypothetical protein FLJ12479 [Homo sapiens]"	RGU34C	29	None detected	RBM15	1p13.3	Putative RNA-binding protein 15 (RNA binding motif protein 15, one-twenty two protein)	"gene ontology : DNA binding, nucleus, RNA binding, cell growth and/or maintenance ; contains 3 RNA recognition motif (RRM) domains"	No	RNA-binding	
rc_AA997773_at	Rattus norvegicus transcribed sequence	RGU34B		Novel gene (sense)	RIN3 (cDNA antisense, gen sense)	14q32.12 (plus strand)	Ras and Rab interactor 3	"gene ontology : neuropeptide signaling pathway ; contains 1 Ras-associating domain and 1 VPS9 domain"	No	Signaling molecule	
rc_AI008087_i_at	transforming growth factor, beta 3	RGU34D		TGF3_RAT	TGFB3	14q24.3	Transforming Growth Factor beta 3 precursor (TGF-Beta 3)	Secreted. Involved in embryogenesis and cell differentiation	Yes in theory, but not worth taking into account (frontotemper	Signaling molecule	

									al dementia)		
rc_AI010476_at	Rattus norvegicus transcribed sequence with strong similarity to protein sp:P15153 (H.sapiens) RAC2_HUMAN Ras-related C3 botulinum toxin substrate 2 (p21-Rac2) (Small G protein) (GX)	RGU34 B		EST matches with gen seq about 400bp away from a novel gene (mouse & human homolog)	RAC2	22q13.1	Ras-related C3 botulinum toxin substrate 2 (P21-RAC2, small G protein, GX)	Cytoplasmic, membrane-associated when activated. Seems to be involved in the regulation of the NADPH oxidase	Yes (SCZD4)	Signaling molecule	
rc_AA963190_s_at	spinophilin	RGU34 B		NEB2_RAT (antisense)	PPP1R9B	17q21.33	Protein phosphatase 1, regulatory subunit 9B, neurabin II, spinophilin	gene ontology : transporter activity, transport, intracellular signaling cascade, membrane	No (NSF on locus 17q21-q22)	Signaling molecule	
rc_AI007768_at	protein phosphatase 1, regulatory (inhibitor) subunit 14a	RGU34 B	22	NM_130403	PPP1R14A (cDNA antisense, gen sense)	19q13.2	Protein phosphatase 1, regulatory (inhibitor) subunit 14A, 17-KDa PKC-potentiated inhibitory protein of PP1	Inhibitor of protein phosphatase 1	No, but coincides with APOE locus	Signaling molecule	
rc_AI111441_at	Rattus norvegicus transcribed sequence with moderate similarity to protein pir:I60307 (E. coli) I60307 beta-galactosidase, alpha peptide - Escherichia coli	RGU34 C	28	RALA_MOUSE	RALA (cDNA antisense, gen sense)	7p14.1	RAS-related protein RAL-A	Belongs to the small GTPase superfamily. RAS family. Gene ontology : RAS small monomeric GTPase activity, protein binding, GTP binding, chemotaxis, signal transduction, small GTPase mediated signal transduction	No (NPY on 7p15.1)	Signaling molecule	
R46930_s_at	phosphatidylinositol 4-kinase	RGU34 C		NM_031083	PIK4CB	1q21.3	Phosphatidylinositol 4-kinase, catalytic, beta polypeptide	gene ontology : inositol/phosphatidylinositol kinase activity, 1-phosphatidylinositol 4-kinase activity, phosphatidylinositol biosynthesis, receptor mediated endocytosis, signal transduction, endosome	Yes (1q21-22 : one of the three best-supported regions cited by O'Donovan et al, Hum Mol Genet, 2003)	Signaling molecule	Yes ?
rc_AI229482_at	myosin VIIA and Rab interacting protein	RGU34 C	-22	None detected	MYRIP (cDNA antisense, gen sense)	3p22.1	Myosin VIIA and RAB interacting protein, RAB effector MYRIP, SLP homologue	unknown	No	Signaling molecule	

							lacking C2 domains, synaptotagmin-like potein homologue E lacking C2 domains-C, exophilin, exophilin 8				
rc_AI234108_at	Rattus norvegicus transcribed sequence with strong similarity to protein pir:JC2472 (H.sapiens) JC2472 brain and reproductive organ-expressed protein - human	RGU34 C		Novel gene	BRE	2p23.2	Brain and reproductive organ-expressed (TNFRSF1A modulator)	gene ontology : signal transduction	No	Signaling molecule (brain)	Yes ?
rc_AI058760_at	No description	RGU34 B		Novel gene	HOXC9	12q13.13	Homeobox protein HOX-C9 (HOX-3B)	Nuclear. Sequence-specific transcription factor which is part of a developmental regulatory system that provide cells with specific positional identities on the anterior-posterior axis	No	Transcription factor	
rc_AA956527_at	forkhead box D4	RGU34 B		FXD4_RAT	FOXD4	9p24.3	Forkhead box protein D4 (Forkhead-related protein FKHL9, Forkhead-related transcription factor 5 (FREAC-5), Myeloid factor-alpha)	"gene ontology : transcription factor activity, regulation of transcription, development, nucleus ; contains 1 fork-head domain"	No	Transcription factor	
rc_AA957545_g_at	T-box 3 (ulnar mammary syndrome)	RGU34 B		Novel gene	TBX3	12q24.21	T-Box transcription factor TBX3 (T-Box protein 3)	Nuclear (potential). Transcriptional repressor involved in developmental processes. Probably plays a role in limb pattern formation	No (DAO on locus 12q24)	Transcription factor	
rc_AI145387_at	Rattus norvegicus transcribed sequences	RGU34 C		Novel gene	FBXL3A	13q22.3	F-BOX and leucine-rich repeat protein 3A, F-BOX protein FBL3A	gene ontology : ubiquitin-protein ligase activity, protein ubiquitination, ubiquitin ligase complex	No	Transcription factor	
rc_AA858921_at	Rattus norvegicus transcribed sequences	RGU34 C	-34	None detected	MEF2C	5q14.2	Myocyte-specific enhancer factor 2C	Nuclear. Transcription factor which binds specifically to the MEF2 element present in the regulatory regions of	No	Transcription factor	Yes

								many muscle-specific genes. Activates transcription via this element. May be involved in myogenesis, neurogenesis and in the development of cortical architecture. Expression is highest during the early stages of postnatal development, at later stages levels greatly decrease. Belongs to the MADS domain family of transcription factors (MEF2 subfamily)			
rc_AA998110_at	Rattus norvegicus transcribed sequence with weak similarity to protein ref|NP_057158.1 (H.sapiens) CGI-143 protein [Homo sapiens]	RGU34B		Novel gene (sense)	Q9H3K6 (cDNA antisense, gen sense)	16p11.2 (minus strand)	MY015 protein	gene ontology : transcription regulator activity	No	Transcription regulator	
rc_AI011382_s_at	Rattus norvegicus transcribed sequence with moderate similarity to protein pir:A30988 (H.sapiens) A30988 lyl-1 protein - human	RGU34B		Novel gene	LYL1	19p13.2	LYL-1 protein (Lymphoblastic leukemia derived sequence 1)	Nuclear (potential). Belongs to the basic helix-loop-helix (BHLH) family of transcription factors, efficient DNA binding requires dimerization with another BHLH protein	No	Transcription regulator	
rc_AA957064_g_at	paired mesoderm homeobox 1	RGU34B		PMX1_MOUSE	PMX1	1q24.2	Paired mesoderm homeobox protein 1 (PRX-1, homeobox protein PHOX1)	Nuclear. Acts as a transcriptional regulator of muscle creatine kinase (MCK) and so has a role in the establishment of diverse mesodermal muscle types. Protein binds to an A/T-rich element in the muscle creatine enhancer (by similarity). Belongs to the paired homeobox family	No (1q21-22 : one of the three best-supported regions cited by O'Donovan et al, Hum Mol Genet, 2003)	Transcription regulator	
rc_AI072812_at	Rattus norvegicus transcribed sequence with strong similarity to	RGU34C		Novel gene	NM_006530	12q15	Glioma-amplified sequence-41, NUMA binding protein 1 (GAS41)	gene ontology : transcription factor activity, regulation of transcription, oncogenesis,	No	Transcription regulator	

	protein ref:NP_006521.1 (H.sapiens) glioma-amplified sequence-41 [Homo sapiens]							nucleus			
re_AA924880_at	Rattus norvegicus transcribed sequence with strong similarity to protein pir:T00379 (H.sapiens) T00379 KIAA0640 protein - human (fragment)	RGU34 B		None detected	Q9P061	11p15.4	No description, protein family:ambiguous	gene ontology : calcium ion binding	No (DRD4 on locus 11p15.5)	unrelated function	
re_AA956968_at	germinal histone H4 gene	RGU34 B		H4_HUMAN	HSTH4J (cDNA antisense, gen sense)	6p22.1 (plus strand)	Histone H4	The nucleosome is an octamer containing two molecules each of H2A, H2B, H3 and H4. The octamer wraps approximately 146 BP of DNA	Yes (6p24-22 : one of the three best-supported regions cited by O'Donovan et al, Hum Mol Genet, 2003)	unrelated function	
re_AI010339_r_at	Rattus norvegicus transcribed sequence with weak similarity to protein ref:NP_057223.1 (H.sapiens) 16.7Kd protein [Homo sapiens]	RGU34 B		Novel gene (sense)	Q8WYQ3	22q11.23	N27C7-4 protein	unknown	Yes (22q11-12 : cited by O'Donovan et al, Hum Mol Genet, 2003)	unrelated function	
re_AA924748_at	vesicle-associated membrane protein 5	RGU34 B		VAM5_RAT	VAMP5	2p11.2	Vesicule-associated membrane protein 5 (VAMP-5, Myobrevin)	Type IV membrane protein (probable). May participate in trafficking events that are associated with myogenesis, such as myoblast fusion and/or glut4 trafficking	No	unrelated function	
re_AI011203_at	Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_079105.1 (H.sapiens) hypothetical	RGU34 B		Novel gene	NM_024829	12p13.1	No description, Protein family:ambiguous	unknown	No	unrelated function	

	protein FLJ22662 [Homo sapiens]										
rc_AI070110_at	Rattus norvegicus transcribed sequences	RGU34B		Novel gene	C1RF_HUMAN	17q21.31	C1Q-related factor precursor	"gene ontology : molecular_function unknown, locomotory behavior, cellular_component unknown ; contains 1 collagenous domain and 1 C1Q domain ; tissue specificity : expressed in brainstem"	No (NSF on locus 17q21-q22)	unrelated function	
rc_AI012085_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_057512.1 (H.sapiens) NESH protein [Homo sapiens]	RGU34B		Novel gene (sense)	NM_016428 (cDNA antisense, gen sense)	17q21.32 (plus strand)	NESH protein, new molecule including SH3 (NESH)	unknown	No (NSF on locus 17q21-q22)	unrelated function	
rc_AI045572_f_at	No description	RGU34B		Novel gene	FKBP11	12q13.12	FK506 binding protein 11 precursor (EC 5.2.1.8, peptidyl-prolyl cis- trans isomerase, ppiase, rotamase)	PPIases accelerate the folding of proteins during protein synthesis	No	unrelated function	
rc_AI012397_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_057143.1 (H.sapiens) peptidylprolyl isomerase (cyclophilin)-like 1 [Homo sapiens]	RGU34B		None detected	PPIL1	6p21.2	Peptidyl-prolyl cis-trans isomerase like 1 (EC 5.2.1.8, ppiase, rotamase, CGI-124)	PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (by similarity)	No, but 6p24-22 : one of the three best-supported region cited by O'Donovan et al, Hum Mol Genet, 2003	unrelated function	
rc_AI059102_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_075809.1 (M.musculus) TNF intracellular domain-interacting protein [Mus musculus]	RGU34B		Novel gene	NM_016274 (cDNA antisense, gen sense)	1q21.2	CK2 interacting protein 1, HQ0024C protein	unknown	Yes (1q21-22 : one of the three best-supported regions cited by O'Donovan et al, Hum Mol Genet, 2003)	unrelated function	

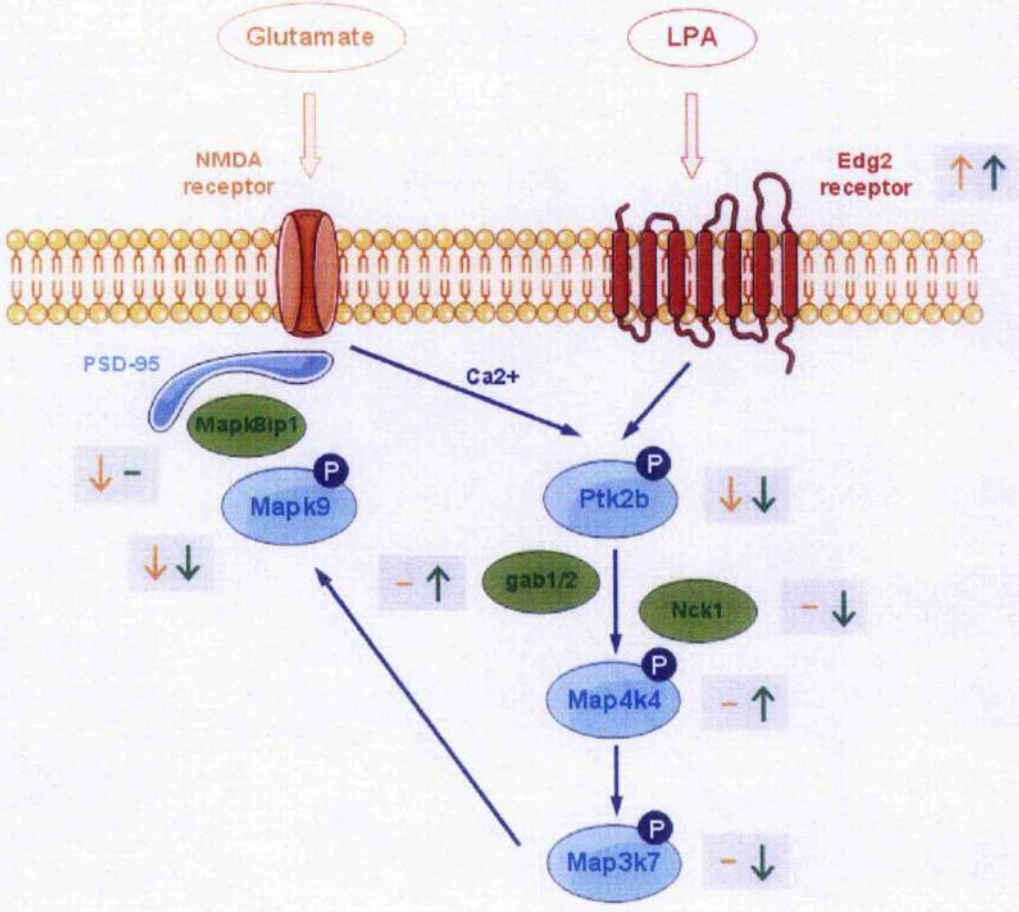
rc_AA849533_at	D123 gene product	RGU34B		NM_053877	C10orf7	10p13	D123 gene product	unknown	Yes (10p15-p11 : cited by O'Donovan et al, Hum Mol Genet, 2003)	unrelated function	
rc_AA956330_at	Rattus norvegicus transcribed sequence with weak similarity to protein sp:P14770 (H.sapiens) GPIX_HUMAN Platelet glycoprotein IX precursor (GPIX) (CD42A)	RGU34B		Novel gene (sense)	GP9	3q21.3	Platelet glycoprotein IX precursor (GPIX, CD42A)	Type I membrane protein. The GPIB-V-IX complex functions as the Von Willebrand factor receptor and mediates Von Willebrand factor-dependent platelet adhesion to blood vessels. The adhesion of platelets to injured vascular surfaces in the arterial circulation is a critical initiating event in hemostasis. GPIX may provide for membrane insertion and orientation of GPIB (with which it is complexed via a non covalent linkage)	No	unrelated function	
rc_AA997670_at	Rattus norvegicus transcribed sequence with strong similarity to protein pir:G02273 (H.sapiens) G02273 LIV-1 protein - human	RGU34B		Novel gene	NM_012319	18q12.2	LIV-1 protein, estrogen regulated (SLC39A6)	gene ontology : metal ion transporter activity, metal ion transport, membrane	No	unrelated function	
rc_AI137430_at	hepatoma-derived growth factor, related protein 2	RGU34C		None detected	NM_032631	19p13.3	No description, protein family:hepatoma derived Growth Factor HDGF	unknown	No	unrelated function	
rc_AI170666_at	Rattus norvegicus transcribed sequence with strong similarity to protein sp:P55145 (H.sapiens) ARGR_HUMAN Arginine-rich protein	RGU34C		Novel gene	ARMET	5p22.31	ARMET protein precursor (arginine-rich protein)	gene ontology : oncogenesis	No	unrelated function	
rc_AI145007_at	Rattus norvegicus transcribed sequence with strong similarity to	RGU34C	20	EST matches with gen seq about 8.3kb away from rat homolog of human	TM4SF12	7q31.31	Tetraspan Net-2	Integral membrane protein (Probable). Belongs to the tetraspanin (TM4SF) family	No	unrelated function	Yes

	protein sp:O95859 (H.sapiens) TNT2_HUMAN Tetraspan NET-2			gene (novel gene)							
rc_AI102258_at	Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_065580.1 (M.musculus) hypothetical protein, 154 [Mus musculus]	RGU34 C		None detected	NM_020686	16p13.2	NPD009 protein	unknown	No	unrelated function	
rc_AI136114_at	Rattus norvegicus transcribed sequences	RGU34 C		Novel gene	Q8IY49	7p22.1	Similar to monocyte to macrophage differentiation-associated (fragment)	gene ontology : cytolysis, integral to membrane	No	unrelated function	
rc_AI145682_at	Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_008854.2 (H.sapiens) stromal cell-derived factor 2 precursor [Homo sapiens]	RGU34 C		Novel gene	SDF2 (cDNA antisense, gen sense)	17q11.2 (minus strand)	Stromal cell-derived factor 2 precursor (SDF-2)	Secreted (Probable). Belongs to the glycosyltransferase family 39. Contains 3 MIR domains	No	unrelated function	
rc_AI234847_at	metastatic lymph node 51	RGU34 C		NM_147144	ML51_HUMAN	17q21.1	MLN51 protein	gene ontology : molecular_function unknown, biological_process unknown, cellular_component unknown	Yes in theory, but not worth taking into account (frontotemporal dementia)	unrelated function	
rc_AI145512_at	Myelin basic protein	RGU34 C	22	MBP_RAT	MBP	18q23	Myelin basic protein (MBP, Myelin A1 protein, myelin membrane encephalitogenic protein)	Cytoplasmic side of myelin. MBP isoforms (4-14) are with PLP the most abundant protein components of the myelin membrane in the CNS. They have a role in both its formation and stabilization. The smaller isoforms might have an important role in	No	unrelated function (neuronal-specific)	Yes

								remyelination of demuded axons in multiple sclerosis. The non- classic group of MBP isoforms (isoforms 1-3/Golli-MBPs) may preferentially have a role in the early developing brain long before myelination, maybe as components of transcriptional complexes, and may also be involved in signaling pathways in T- cells and neural cells			
:c_AI009300_at	preproenkephalin, related sequence	RGU34B		PENK_RAT	PENK	8q12.1	Proenkephalin A precursor (contains : synenkephalin, met-enkephalin) (Opioid growth factor, OGF)	Secreted. Met-and Leu-enkephalins compete with and mimic the effects of opiate drugs. They play a role in a number of physiologic functions, including pain perception and responses to stress	No	unrelated function (role in CNS)	Yes

Appendix C.

Schematic representation of the functional relationship between Edg2 and "Pyk/Nck" pathway genes and microarray gene expression changes in the chronic PCP model of schizophrenia (RG-U34A, B and C GeneChip® data, Catherine Winchester) and in dorso-lateral prefrontal cortex from schizophrenic patients (HG-133A GeneChip® data, Hiromitsu Ozeki)



The "Pyk/Nck" pathway is structurally organised as a MAPK signalling cascade whereby the MAP kinase Mapk9 (Jnk2) is activated by the dual specificity MAP kinase kinases Map3k7/Map4k4 which are themselves activated by MAP kinase kinase kinases downstream from the protein tyrosine kinase Ptk2B. Gab1/2 and Nck1 are scaffold proteins that bring upstream and downstream kinases in close proximity ensuring effective, specific signal transduction.

Direction of expression changes of "Pyk/Nck" genes detected by microarray analysis of the prefrontal cortex of rats treated with PCP according to the chronic PCP model treatment regime (Cochran *et al.* 2003) and in post-mortem dorso-lateral prefrontal cortex from schizophrenic patients are shown in grey boxes located beside each gene, orange arrows representing rat microarray changes and green arrow human microarray changes. Horizontal dashes signify that no significant change in expression was detected, either in the rat microarray (orange dash) or in the human microarray (green dash).

Appendix D.

Further details on statistical analysis of SNP genotyping experiments

1. Tests for deviation from Hardy-Weinberg equilibrium

Exceptions (non-equilibrium)

- Mutation
- Gene migration
- Genetic drift
- Non-random mating
- Natural selection

2. Tests for association (adapted from Sasieni PD, 1997).

n11(e): Genotype 11 (expected)
n12(e): Genotype 12 (expected)
n22(e): Genotype 22 (expected)
f a1: Frequency of allele 1 +/- standard deviation
F: Inbreeding coefficient
p (Pearson): Pearson's goodness-of-fit chi-square (degree of freedom = 1)
p (Llr): Log likelihood ratio chi-square (degree of freedom = 1)
p (Exact): Exact test
The following equations correspond to risk allele 2.
Odds ratio (allele freq. difference): (Case_a2 * Control_a1) / (Case_a1 * Control_a2)
Chi2 (allele freq. difference): (P) = Pearson's goodness-of-fit chi-square (df=1), (F) = Fisher's exact test
Odds ratio (heterozygous): (Case_12 * Control_11) / (Case_11 * Control_12)
Odds ratio (homozygous): (Case_22 * Control_11) / (Case_11 * Control_22)
Odds ratio (allele positivity): ((Case_12+Case_22) * Control_11) / (Case_11 * (Control_12+Control_22))
Common odds ratio: (Case_12*Control_11/N01 + Case_22*Control_12/N12 + 4*(Case_22*Control_11/N02))/(Case_11*Control_12/N01 + Case_12*Control_22/N12 + 4*(Case_22*Control_11*Case_11*Control_22)**0.5/N02)

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