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(54) Title: NOVEL SCHIZOPHRENIA ASSOCIATED GENES

(57) Abstract: The present invention relates to the identification of genes involved and/or associated with schizophrenia and related diseases, as well as particular identified genes and their potential use in therapy/diagnosis of schizophrenia and related diseases.

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NOVEL SCHIZOPHRENIA ASSOCIATED GENES

Field of the Invention

The present invention relates to the identification of genes involved and/or associated with schizophrenia and related diseases, as well as particular identified genes and their potential use in therapy/diagnosis of schizophrenia and related diseases.

Background to the Invention

Schizophrenia is a devastating mental illness which affects 1% of the world population, the aetiology of which remains elusive. There is known to be a strong genetic component to the disease, with incidence rising to between 10% and 49% in those with close relatives suffering from the disease. However, to date, there is a poor understanding of the genes involved.

Schizophrenia is characterised by positive symptoms (such as hallucinations and delusions), negative symptoms, (such as social withdrawal, anhedonia and self-neglect) and cognitive deficits (including impairments in executive function and attention). The majority of patients exhibit the first psychotic episode at around 16 – 26 years of age, and thereafter are unlikely ever to make a full recovery. Most patients remain considerably impaired for the rest of their lives, and a high proportion commit suicide, despite treatment with current antipsychotic medication.

A few specific neurobiological features have been identified in the CNS of patients with schizophrenia. As compared to control subjects, schizophrenic patients exhibit a lower metabolic activity in the prefrontal cortex when performing a cognitive task (Tamminga et al., 1992). This metabolic "hypofrontality", generally

accompanied by parallel alterations in metabolic activity in the temporal cortex and hippocampus, is highly characteristic of schizophrenia. In addition, post-mortem studies have revealed a selective loss of a particular population of inhibitory interneurons – parvalbumin-containing GABAergic interneurons (axo-axonic or "chandelier" cells) – in the prefrontal cortex and hippocampus from patients with schizophrenia (Beasley & Reynolds, 1997; Lewis et al., 1999). This regionally specific pathology focuses attention on the prefrontal cortex as a major site of dysfunction in schizophrenia, and is consistent with evidence that many of the cognitive tasks where patients show deficits are dependent on prefrontal cortex function. In addition, the severity of negative symptoms and cognitive deficits has been shown to correlate with the degree of metabolic hypofrontality in the prefrontal cortex (Hill et al., 1994; Wolkin et al., 1985; Wolkin et al., 1992).

Existing antipsychotic drugs show considerable efficacy against the positive symptoms, but are relatively ineffective against the negative symptoms and cognitive deficits, which arguably represent the major long-term barrier to the patients resuming something that approaches a normal lifestyle (Goldberg et al., 1993). In addition, all current anti-psychotic drugs display a variety of unpleasant side-effects, including parkinsonism, dysphoria, weight-gain, hypotension or sedation. One of the major goals of modern drug development is to produce a novel antipsychotic drug which is more effective in ameliorating the negative symptoms and cognitive deficits characteristic of schizophrenia than existing therapies. The development of improved antipsychotic drugs which will have superior action against the negative symptoms and cognitive dysfunction has been severely hampered by the lack of knowledge of which genes are involved and/or associated with schizophrenia. The hope is that an understanding of the causes of schizophrenia will ultimately lead to markedly

improved drug therapies aimed at curing the disease rather than treating some of the symptoms.

The discovery of novel therapeutic strategies for treating schizophrenia has received a dramatic boost from the recent discovery of a viable animal model of the disease (Cochran et al., 2003; Morris et al., 2005). The model exploits the clinical properties of the psychotogenic drug phencyclidine (PCP). When PCP is chronically abused by humans, it induces a range of symptoms virtually indistinguishable from schizophrenia, including positive and negative symptoms, and also cognitive deficits (Allen & Young, 1978). Since PCP is an antagonist of the NMDA class of glutamate receptor (Morris et al., 2005), this has led to intense interest in the possibility that glutamatergic hypoactivity might contribute to the aetiology of schizophrenia (Tamminga, 1998). Indeed, evidence that the levels of NMDA receptors are increased in the cortex of patients with schizophrenia tends to support this view (Dracheva et al., 2001; Ishimaru et al., 1994). In the animal model of schizophrenia, rats are treated chronically with a specific dose of PCP. The treatment induces metabolic hypofrontality, altered hippocampal metabolic activity, chandelier cell dysfunction, including reduced parvalbumin expression, and deficits in prefrontal cortex-dependent cognitive tasks which have been designed to mirror those where schizophrenic patients show deficits (Morris et al., 2005). Thus this animal model accurately reproduces in rats the clinical profile of the disease in humans.

Little is known about the genes that cause schizophrenia, or more specifically any alteration of expression/mutation of genes in a patient suffering from schizophrenia.

It is amongst the objects of the present invention to identify one or more genes, the altered expression of which are associated with schizophrenia.

Summary of the Invention

The present invention is based on experiments which were designed to identify genes potentially contributing to the aetiology of schizophrenia, using prefrontal cortex tissue from rats treated according to the model of Cochran et al (2003), and post-mortem prefrontal cortex tissue from patients with schizophrenia, and controls.

In certain embodiments, the present invention relates to the differential expression of certain genes, notably certain kinases, and how proteins expressed from these genes, or the differential expression of these genes, may be used to screen for compounds which may modulate the activity or the expression of the differentially expressed genes, or measure an effect a compound may have on the expression of such differentially expressed genes in an animal model of schizophrenia. It may also be possible to use the information as a prognostic or diagnostic marker for schizophrenia.

In a first aspect there is provided a method of screening a compound for treating schizophrenia, which comprises:

(a) bringing a test compound into contact with a wild type cell or transformed cell which is capable of expressing one or more schizophrenia-associated genes selected from the group consisting of PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1;

(b) detecting a level of expression or activity of said one or more schizophrenia-associated gene(s)/protein(s) in said cell; and

(c) selecting a compound which promotes or suppresses expression or activity of the schizophrenia-associated gene/protein in comparison with a control (vehicle).

There is also provided a method for measuring an anti-schizophrenia effect of a compound using an animal model of schizophrenia, which comprises:

(a) administering the compound to an animal model displaying characteristics of schizophrenia;

(b) detecting a level of expression or activity of one or more genes/proteins selected from the group consisting of PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1;

(c) comparing the level of expression or activity of said one or more genes/proteins with a level of expression or activity of said one or more genes/proteins in a further control animal displaying characteristics of schizophrenia, but which has not been administered the compound; and

(d) selecting compounds for further evaluation as potential anti-schizophrenia agents, which modulate expression or activity of said one or more genes/proteins, in comparison to the control animal.

It is to be understood that the term schizophrenia as used herein, relates to schizophrenia and other related disorders such as bipolar affective disorder, brief psychotic disorder, schizophrenia form disorder, schizopffective disorder and the like.

The term "expression" means allowing or causing the information in a gene or DNA sequence to become manifest, for example, by producing mRNA or a protein. The level of "expression" can easily be detected by methods known to the skilled

addressing including hybridisation studies, quantitative PCR, northern and western blotting, techniques and immunoassays. See for example Sambrook et al, 2000.

It may also be appropriate to detect a level of expression of variant of the gene, which may lead to an alteration in the level of activity of the resulting protein.

Allelic variants are usually observed by comparing their nucleotide or (in the case of variant polypeptides) amino acid sequences to a common "wild-type" or "reference" sequence. Thus, a "wild-type" or "reference" allele of a gene refers to that allele of a gene having a genomic sequence designated as the wild-type sequence and/or encoding a polypeptide having an amino acid sequence that is also designated as a wild-type sequence. The wild-type allele may be arbitrarily selected from any of the different alleles that may exist for a particular gene. However, the allele is most typically selected to be the allele which is most prevalent in a population of individuals.

The level of activity of the protein may be the ability of the protein to interact with a different protein or ligand and this can be detected by enzyme assays and ligand/protein binding assays, known in the art.

For example, JNK activity is typically measured by directly monitoring the amount of phosphorylated JNK in a tissue or cell extract using phosphorylation-state-specific antisera (e.g. Mizuno et al., 2002; Machida et al., 2004). Alternatively a tissue or cell extract is incubated with c-jun or ATF3 protein, or with synthetic peptides corresponding to the sites of phosphorylation of these proteins, either in the presence of ^{32}P -ATP, so that the incorporation of ^{32}P into the c-jun or ATF3 protein can be monitored, or in the presence of unlabelled ATP, in which case the levels of phosphorylated c-jun or ATF3 are monitored via phosphor-specific antisera for these proteins (e.g. Poitras et al., 2003). Similarly, MAP2K7 activity is typically measured

by directly monitoring the amount of phosphorylated MAP2K7 in a tissue or cell extract using phosphorylation-state-specific antisera. Alternatively a tissue or cell extract is incubated with JNK1 or JNK2, along with c-jun or ATF3 protein, or with synthetic peptides corresponding to the sites of phosphorylation of these proteins, either in the presence of ^{32}P -ATP, so that the incorporation of ^{32}P into the c-jun or ATF3 protein can be monitored, or in the presence of unlabelled ATP, in which case the levels of phosphorylated c-jun or ATF3 are monitored via phosphor-specific antisera for these proteins (e.g. Fleming et al., 2000). MAP3K7 activity can be measured by directly monitoring the amount of phosphorylated MAP3K7 in a tissue or cell extract using phosphorylation-state-specific antisera. Alternatively a tissue or cell extract is incubated with MKK7, or with synthetic peptides corresponding to the sites of phosphorylation of this protein, either in the presence of ^{32}P -ATP, so that the incorporation of ^{32}P into the MKK7 protein can be monitored, or in the presence of unlabelled ATP, in which case the levels of phosphorylated MKK7 are monitored via phosphor-specific antisera. Activity of MAP4K4 or TNIK can be measured by directly monitoring the amount of phosphorylated MAP3K7 in a tissue or cell extract using phosphorylation-state-specific antisera. Activity of MAP4K4 or TNIK can also be assessed by incubating a tissue or cell extract with MAP3K7, and monitoring the activity of MAP3K7 as above (eg Yao et al., 1999; Fu et al., 1999). PAK1 activity can be measured by directly monitoring the amount of phosphorylated PAK1 in a tissue or cell extract using phosphorylation-state-specific antisera (e.g. Koh et al., 2002). Alternatively a tissue or cell extract is incubated with tyrosine hydroxylase or myelin basic protein, or with synthetic peptides corresponding to the sites of phosphorylation of these proteins, either in the presence of ^{32}P -ATP, so that the incorporation of ^{32}P into the tyrosine hydroxylase or myelin basic protein can be

monitored, or in the presence of unlabelled ATP, in which case the levels of phosphorylated tyrosine hydroxylase or myelin basic protein are monitored via phosphor-specific antisera (e.g. Poitras et al., 2003). CamKIV activity can be measured by directly monitoring the amount of phosphorylated CamKIV in a tissue or cell extract using phosphorylation-state-specific antisera. Alternatively a tissue or cell extract is incubated with CREB or CamKII-gamma, or with synthetic peptides corresponding to the sites of phosphorylation of CREB or CamKII-gamma, either in the presence of ^{32}P -ATP, so that the incorporation of ^{32}P into the CREB or CamKII-gamma can be monitored, or in the presence of unlabelled ATP, in which case the levels of phosphorylated CREB or CamKII-gamma are monitored via phosphor-specific antisera (e.g. Enslen et al., 1996). PPM1E and PPM1F activity can be measured by directly monitoring the amount of phosphorylated PPM1E or PPM1F in a tissue or cell extract using phosphorylation-state-specific antisera. Alternatively a tissue or cell extract is incubated with PAK1, CamKIV or CamKII, which have been previously phosphorylated with ^{32}P derived from ^{32}P -ATP via the action of CaMK kinase, or with synthetic phosphor-peptides corresponding to the sites of phosphorylation of PAK1, CamKIV or CamKII. The release of ^{32}P can then be monitored, or alternatively the reduction in the levels of phosphorylated PAK1, CamKIV or CamKII are monitored via phospho-specific antisera (e.g. Koh et al., 2002; Tan et al., 2001).

Detection of protein may typically be carried out using labelling of the protein with an appropriate marker molecule. For example a labelled or unlabelled antibody or other binding agent specific for the particular protein may be used, to bind the protein and allow its detection. If an unlabelled antibody or other binding agent is used, it will be necessary to employ a labelling agent designed to bind to the antibody

or binding agent, in order to allow detection. It may also be necessary to first permeabilise or lyse the cells in order to allow the proteins to be detected and many techniques for achieving this are known to the skilled man. Moreover, as some of the proteins identified herein are kinases, antibodies or specific binding agents which are able to bind to the phosphorylated or non-phosphorylated forms of the kinases substrate, may be used, in order to detect a level of kinase activity.

As described in more detail hereinafter, the inventors have identified a number of associated genes/proteins (PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1) which may be differentially expressed in a schizophrenic subject. Thus, detection of the level of expression or activity of one or more of these genes/proteins in a cell or animal, in comparison to a control sample, may be used to screen compounds for potential use as anti-schizophrenia drugs.

Generally speaking, the more genes/proteins which are included in any test may lead to an improved test. Thus, the method may preferably include at least 2, 3, 4, 5, 10, 15 or all of the aforementioned genes identified herein. Preferred sets of genes, the differential expression of which, whether up or down with respect to a control, are identified in Tables 1 and 2 and a particularly preferred sub-set of genes comprise one or more of the MAP kinases identified (e.g. MAP2K7, MAPK9, MAP3K7 and/or MAP4K4).

The present invention also provides methods for identifying, evaluating, and/or monitoring drug candidates for the treatment of schizophrenia. According to the invention, a candidate drug may be assayed for its ability to modulate the expression or activity of one or more of the genes/proteins identified herein. For

example, a specific drug may increase or decrease the expression or activity of one or more of the genes/proteins identified herein.

Moreover, there may be existing drugs or chemical entities, such as small molecules or peptides etc. which are already known to have an effect on the expression on one or more of the genes/proteins identified hereinabove, but not in the context of being associated with Schizophrenia. As such, it would not be appropriate to use such drugs/chemical entities as potential anti-Schizophrenia agents.

Thus, in a further aspect the present invention provides use of a agent which is capable of modulating activity or expression of one or more of the genes/proteins identified herein, for the manufacture of a medicament for treating Schizophrenia and/or Schizophrenia associated disorders.

There is also provided a method of treating Schizophrenia and/or Schizophrenia associated disorders comprising the step of administering an agent, to a subject in need thereof, which is capable of modulating the activity or expression of one or more of the genes/proteins identified herein so as to treat Schizophrenia and/or a Schizophrenia associated disorder.

Of course an existing/known chemical entity active on one or more of said genes/proteins could be administered to the described animal model in order to observe and effect on the behaviour of the animal.

It is also possible to evaluate, or study the biological function of the genes identified herein and this can be assessed by utilizing relevant *in vivo* and *in vitro* systems. *In vivo* systems can include, but are not limited to, animal systems which naturally exhibit the symptoms of schizophrenia, or ones which have been developed to exhibit such symptoms, as for example the model described in WO01/75440. Further, such systems can include, but are not limited to transgenic animals systems.

In vitro systems can include, but are not limited to, cell-based systems comprising the identified gene/polypeptide expressing cell types. The cells can be wild type cells, or can be non-wild type cells containing modifications known or suspected of contributing to schizophrenia.

In further characterising the biological function of said identified gene(s), the expression of said identified gene(s) can be modulated within the *in vivo* and/or *in vitro* systems, i.e. either overexpressed or underexpressed in, for example, transgenic animals and/or cell lines, and the subsequent effect on the system can then be assayed.

Alternatively, the activity of the product of the identified gene can be modulated by either increasing or decreasing the level of activity in the *in vivo* and/or *in vitro* system of interest, and its subsequent effect then assayed.

The information obtained through such characterisations can suggest relevant methods for the treatment or control of schizophrenia. For example, relevant treatment can include a modulation of gene expression and/or gene product activity. Characterisation procedures such as those described herein can indicate whether such modulation should be positive or negative. As used herein, "positive modulation" refers to an increase in gene expression or activity of the gene or gene product of interest. "Negative modulation", as used herein, refers to a decrease in gene expression or activity.

For example, it can be seen from the results presented herein, that the identified genes/proteins are differentially expressed (see Table 2), that is, increased or decreased in expression in tissue from schizophrenic patients, as compared to non-schizophrenic patients. It may therefore be appropriate to seek to correct such differential expression by either up-regulating or down-regulating, in some manner, the expression of one or more of said identified genes.

For genes/proteins which are expressed to a lesser degree in schizophrenic patients as compared to non-schizophrenic patients, it may be possible to redress this imbalance using gene therapy techniques, or simply by administering the relevant proteins to the subject. With regards to gene therapy, the skilled addressee is well aware of suitable vector constructs, such as adeno associated virus vector constructs which may be administered to a subject to cause expression of a chosen gene. Appropriate promoters may also be used to control expression of the cloned gene in specific cell types and/or at certain times.

With regards to down-regulation of gene expression, this may be achieved by way of, for example, RNAi techniques known in the art.

It will be appreciated that all such techniques may first be applied to an appropriate schizophrenia animal model, in order to determine their efficacy, before turning to human schizophrenic patients.

In vitro systems can be designed to identify compounds capable of binding said identified gene(s) products of the invention. Compounds identified can be useful, for example, in modulating the activity of wild type and/or mutant gene(s) products, can be useful in elaborating the biological function of said identified gene(s) products, or can disrupt normal identified gene(s) product interactions.

In one embodiment, a candidate drug may be added to cells or sample tissue prior to analysis. Preferred cells are cell lines grown from tissue, particular brain tissue, e.g. prefrontal cortex tissue from schizophrenic subjects. Alternatively, cells isolated from the brain of the PCP rat model described in WO01/75440 may be employed.

In another embodiment, the invention provides screens for a candidate drug which modulates gene expression and/or protein activity of said one or more disclosed genes/proteins in brain tissue, or interferes or enhances the binding of said one or more proteins to its substrate.

The term "candidate drug" or equivalent as used herein describes any molecule, e.g. an antibody or antibody fragment, aptamer protein, oligopeptide, fatty acid, steroid, small organic molecule, polysaccharide, polynucleotide, RNAi molecule, antisense molecule, ligand, bioactive partner and structural analogs or combinations or conjugates thereof, to be tested for candidate drugs that are capable of directly or indirectly altering the activity or the expression of said one or more genes/proteins. Such candidate drugs may also be administered in combination with an agent designed to facilitate entry into a cell and many such agents are known in the art.

In a further aspect there is provided a method of prognosis or diagnosis of schizophrenia comprising the steps of:

- a) providing a tissue or body fluid sample from a test subject;
- b) detecting a level of expression or level of activity of one or more of the following genes/proteins PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1 in a cell or cells from said sample; and
- c) comparing the level of expression or level of activity of said one or more genes/proteins with a level of expression or level of activity of said one or more genes/proteins in a control sample from a subject not suffering or predisposed to suffering from schizophrenia.

Typical body fluid samples include blood, serum, urine, cerebrospinal fluid (CSF), saliva, etc. Suitable tissue samples include buccal cells and skin.

Moreover, a gene sequence may comprise a mutation or polymorphism, with respect to the wild-type sequence and the level of expression of such a mutant or polymorphic sequence may also be detected, by for example, hybridisation, quantitative PCR, electrophoretic mobility, or nucleic acid sequence studies, known in the art.

The term "polymorphism" refers, generally, to the coexistence of more than one form of a gene (e.g., more than one allele) within a population of individuals. The different alleles may differ at one or more positions of their nucleic acid sequences, which are referred to herein as "polymorphic locuses". When used herein to describe polypeptides that are encoded by different alleles of a gene, the term "polymorphic locus" also refers to the positions in an amino acid sequence that differ among variant polypeptides encoded by different alleles.

The polymorphisms of the present invention include "single nucleotide polymorphisms" (SNPs) and microsatellite repeats. The term SNP refers to a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. Typically, the polymorphic site of an SNP is flanked by highly conserved sequences (e.g., sequences that vary in less than 1/100 and, more preferably, in less than 1/1000 individuals in a population). The polymorphic locus of an SNP may be a single base deletion, a single base insertion, or a single base substitution. Single base substitutes are most typical.

Prediction of whether or not a test subject is likely to have or be predisposed to developing schizophrenia, may be based on a profile of expression and this may be carried out using appropriate computer software.

The present invention therefore also provides arrays of gene expression detection agents for use in the method of the present invention.

Thus, in a further aspect, there is provided a DNA array for use in a method according to the present invention the array comprising or consisting essentially of one or more of the following genes or sequence specific fragments thereof: PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1, the array being immobilised on a support.

Preferably the array is a microarray. Advantageously the array or microarray is prepared on any suitable, preferably non-porous substrate. Typically the suitable substrate may include glass or a plastic material. Information regarding suitable substrates and the protocols used to generate arrays or microarrays may be obtained from the National Human Genome Research Institute, Bethesda USA.

Generally the surface of the suitable microarray substrate is treated in some way so that nucleic acid specific to said genes, that is nucleic acid corresponding to said gene or specific fragment thereof may be coupled to it. For example the surface of the suitable substrate may be made hydrophobic so as to prevent spread of individual nucleic acid samples applied to the microarray substrate and positively charged so as to facilitate the coupling of the nucleic acid to the microarray substrates. Such a hydrophobic/positively charged surface may be obtained by use of a substance such as poly-L-lysine.

After such preparation of the microarray substrate, the nucleic acid fragments may be spotted on to the surface as an array. Preferably automated printing procedures known in the art may be utilised to apply the nucleic acid fragments as an array.

Preferred gene expression detection agents hybridise specifically to the genes identified herein whose expression is correlated with having or being disposed to developing schizophrenia. Such agents may be RNA, DNA or PNA molecules. Preferred agents are fragments of the above-identified genes, e.g. oligonucleotides specific therefore. Alternative agents may bind specifically to the protein expression products of the marker genes disclosed herein. Preferred agents include antibodies and aptamers.

Agents, such as oligonucleotides, are preferably attached to a solid support in the form of an array. Oligonucleotide arrays in the form of DNA microarrays and useful hybridisation assays are known in the art and disclosed for example in US Patent Nos. 5,631,734; 5,874,219; 5,861,242; 5,585,659; 5,856,174; 5,843,655; 5,837,832; 5,834,758; 5,770,722; 5,770,456; 5,733,729; 5,556,752; 6,045,996 and 6,261,776. In a preferred embodiment, an array includes oligonucleotides for measuring the expression level of the genes identified herein. However, it is also possible to use cDNAs or PCR products for probes.

The present invention further provides a database of said identified genes and information about the said genes, including the expression levels that are characteristic of schizophrenic and non-schizophrenic subjects. According to the invention, said gene information is preferably stored in a memory in a computer system. Alternatively, the information is stored in a removable data medium such as a magnetic disk, a CDROM, a tape, or an optical disk. In a further embodiment, the input/output of the computer system can be attached to a network and the information about the identified genes can be transmitted across the network.

Preferred information includes the identify of the genes identified herein, the differential expression of which correlates with an expected diagnosis or prognosis to developing schizophrenia. In addition, reference expression levels of said genes may be stored in a memory or on a removable data medium. According to the invention, a reference expression level is a level of expression of the marker gene that is indicative normal, that is, a non-schizophrenia subject and as such can be used as a reference to determine whether or not a test subject's level of expression is significantly increased or decreased with respect to the reference level.

In a highly preferred embodiment, a computer system or removable data medium includes the identity and expression information. In addition, information about expression levels of said genes for non-schizophrenia tissue may be included.

Detailed description of the Invention

The present invention will now be further described by way of example and with reference to the following Figures which show:

Figure 1 shows the relationship between Pyk-Nck-Jnk pathway gene products;

Figure 2 shows the results of RT-qPCR for human post mortem tissue RNAs (two-tailed t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$);

Figure 3 shows the ability of increased Nck 1 gene expression to alter Pyk-Nck-Jnk pathway activity in neuronal cells: Nck1 was over-expressed in NG108-15 cells, and total JNK activity measured by phosphor-JNK ELISA. (one-tailed t-test; * $p < 0.05$);

Figure 4a shows the levels of NMDA receptor NR1 subunit protein, Parvalbumin mRNA, and phospho-JNK activity in prefrontal cortex tissue from mice with a deleted Nck1 gene to render the Pyk-Nck-Jnk2 pathway dysfunctional: (two-tailed t-test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$);

Figure 4b shows reduced metabolic activity in prefrontal cortex (hypofrontality) from mice with a deleted Nck1 gene (Nck1 knockout mice - KO) to render the Pyk-Nck-Jnk2 pathway dysfunctional, as compared to control (wild-type – WT) mice: (two-tailed t-test; *** $p < 0.001$ relative to control);

Figure 4c shows increased metabolic activity in the auditory processing circuit (Auditory Cortex, AuCx; Parasubiculum, PaS; and Inferior Colliculi, IC) from mice with a deleted Nck1 gene to render the Pyk-Nck-Jnk2 pathway dysfunctional, as compared to control (wild-type – WT) mice: (two-tailed t-test; * $p < 0.05$ relative to control);

Figure 5 shows the results of SNP analysis for MAP2K7 in human blood samples from schizophrenic patients and controls. Results are analysed using two complementary statistical tests – Fisher's exact test and the trend test. The genotype is shown on the abscissa, and frequency of detection on the ordinate;

Figure 6 shows the results of SNP analysis for MAP2K7, in pooled data from Glasgow and London blood samples, from schizophrenia patients and controls. Results are analysed using two complementary statistical tests – Fisher's exact test and the trend test. The genotype is shown on the abscissa, and frequency of detection on the ordinate;

Figure 7 shows the interaction of CamKIV with PPM1E and PPM1F in neuronal cells. NG108-15 neuronal cells were transfected with combinations of vectors expressing FLAG-tagged-PPM1E, FLAG-tagged-PPM1F or a constitutively

active truncated mutant of CamKIV (caCamKIV). Following immunoprecipitation of the FLAG tag. CamKIV was detected by western blotting. The presence of a band representing either endogenous CamKIV or transfected caCamKIV indicates a physical interaction with PPM1E/PPM1F. Thus endogenous CamKIV interacts with PPM1F, while active CamKIV interacts with both PPM1E and PPM1F;

Figure 8a and b shows recombinant human PPM1E and PPM1F purified from *E. coli*. Purification of the arrowed His-tagged protein was confirmed by western blotting with peroxidase-conjugated anti-His antibody (data not shown). M, E1 and E2 stand for Molecular weight marker, eluted fraction with 150 mM imidazole and eluted fraction with 200 mM imidazole, respectively;

Figure 9 shows measurement of phosphatase activity of recombinant human PPM1E by BIOMOL GREEN;

Figure 10 shows measurement of phosphatase activity of recombinant human PPM1E by AlphaScreen Phosphosensor kit; and

Figure 11 shows measurement of phosphatase activity of recombinant human PPM1E by the principle of HTRF.

Materials and Methods

Chronic PCP Treatment:-

Male hooded Long Evans rats (approximately 300g at the start of the study) were randomly assigned into two treatment groups: chronic PCP (2.58mg/kg PCP.HCl) and chronic vehicle (saline 1ml/kg). The rats were treated according to the YRING chronic PCP model (Cochran et al., 2003) (i.p. injections once daily for five days, then once every 72 hours for a further 3 weeks) and killed by cervical dislocation 72

hours after the last injection. The brains were removed and the prefrontal cortex dissected.

Microarray analysis:-

Sample information and Preparation of total RNA

-rat tissue:

Qiagen RNeasy Minicolumns were used to isolate total RNA from the 12 chronic PFC tissue samples (PCP n=6, vehicle n=6). 10µg of total RNA was treated with DNaseI to remove genomic DNA and purified using the Qiagen RNeasy Minicolumns. The yield, concentration and integrity of the total RNA were determined using a GeneQuant spectrophotometer, agarose gel electrophoresis and an Agilent 2100 Bioanalyzer (Sir Henry Wellcome Functional Genomics Facility, University of Glasgow, SHWFGF).

-human tissue:

The Scottish Biomedical Foundation provided a set of PFC samples (Brodmann region 10) from controls (n = 12) and schizophrenic patients (n = 12). Total RNA was extracted from approximately 500 mg/sample of DLPFC using Qiagen Lipid Midi Kit (Qiagen). The manufacturer's protocol was modified by adding a centrifugation step after homogenisation to remove the remaining debris and to prevent blocking the purification column. To remove genomic DNA contamination, DNase treatment was performed on the purification column. The quality of the total RNA was assessed using the Agilent 2100 Bioanalyzer. Eighteen high quality RNAs were finally used for the microarray study. The samples – 9 controls (7 males and 2 females) and 9 schizophrenics (7 males and 2 females) – were matched as far as possible using the following information: average post mortem delay 20.1 ± 5.7 h

(control) and $20.3 \pm 8.9\text{h}$ (schizophrenics); average age $58 \pm 14.3\text{yr}$ (control) and $55.2 \pm 16\text{yr}$ (schizophrenics).

Microarray

RG-U34A-C Affymetrix GeneChips were used for the rat chronic PCP microarray study. HG-U133A Affymetrix GeneChips were used for the human post mortem microarray study. Affymetrix protocols were followed for sample labelling. GeneChip hybridisation and scanning.

Microarray data analysis

RMA (Robust Multi-Array Average Expression Measure) was carried out (MBSU, University of Glasgow) to perform a low level analysis for the HG-U133A/RG-U34 GeneChip data using BioConductor software. RMA normalises the signal intensities across the GeneChips by adjusting the fluorescent strength of each array:

1. Probe-specific background was corrected to compensate for non-specific binding using PM (Perfect Matching signal) distribution rather than PM-MM (Mismatching signal) values;
2. Probe-level multichip quantile normalisation was performed to unify PM distributions across all GeneChips; and
3. Robust probe-set summary of the log-normalised probe-level data by median polishing.

SAM (Significance Analysis of Microarrays) (Tusher et al., 2001) is software for performing a high level analysis on the normalised RMA data to identify differentially expressed genes. It uses repeated permutations of the data to determine if the expression of any genes is significantly related to schizophrenia. The cut-off for

significance is determined by the user based on the false positive rate. One can also choose a fold change parameter, to ensure that called genes change at least by a pre-specified amount. For comparison, a conventional t-test was used as an alternative method to assess the level of significance for differentially-expressed genes. The p-value for cut off of non-altered genes was set above 0.050. However with this value 5% of risk will be detected as nonconfidence by chance.

Real-time quantitative PCR (RT-qPCR):-

First strand cDNAs were generated by reverse transcription using 2 µg of human or rat prefrontal cortex total RNA used for the microarray study, SuperScript II RNaseH- Reverse Transcriptase (Invitrogen) and a Hexanucleotide Mix (Roche) in a reaction volume of 20 µl. This 1st strand cDNA synthesis was diluted 1/20 to a final volume of 400 µl and then 2.5 µl was amplified using Universal Master Mix (ABI), gene-specific Assays-on-Demand (ABI) and the SDS7000 (ABI). Two methods of statistics were used to analyse the RT-qPCR data to identify differentially expressed genes: ANCPVA and comparative C_T method, followed by a two-tailed t-test. *GAPDH* was used as the housekeeping gene (internal control).

NCK1 over-expression in NG108-15 cells:-

NG108-15 cells were plated at a cell density of 2×10^5 cells in 1 ml DMEM (Gibco) and 10 % FBS (Gibco) without antibiotic into two 12 well plates and allowed to grow overnight at 37°C, 5 % CO₂. DNA was diluted in 100 µl of serum free DMEM and mixed gently. Lipofectamine 2000 reagent (Invitrogen) was mixed before use and diluted in 100 µl of serum free DMEM and mixed gently. The reagent was incubated at room temperature for 5 minutes. The diluted DNA was then added

to the diluted lipofectamine, mixed gently and incubated at room temperature for 20 minutes. Fresh medium was then added to the cells and the 200 μ l DNA/lipofectamine mix was placed onto the cells. The cells were incubated for 48 hours at 37°C, 5 % CO₂ and the protein harvested. The over-expression was assayed using western blots with antibodies to NCK1 (Santa cruz) and FLAG (Sigma). 10 μ g of protein was loaded in each well of an SDA-PAGE gel (10 %) when using the FLAG antibody and 30 μ g of protein was loaded when using the NCK1 antibody. The various conditions tested are indicated in the results.

Nck1 -/- KO mice and ICR +/- mouse cortex samples:-

The cortex was dissected from 6 *Nck1* -/- KO and 6 ICR +/- wild-type control mice.

Western blots of Nck1 KO -/- mice: =

Whole cell lysates were prepared from the cortex of 6 *Nck1* -/- KO and 6 ICR +/- wild-type control mice using cell lysate buffer containing proteinase and phosphatase inhibitors. The soluble protein was isolated by centrifugation and quantified using the Bradford method. 30 μ g of protein was electrophoresed in a polyacrylamide gel and transferred by western blotting to PVDF membrane. A NCK1 antibody (Chemicon, 1:10,000) was used to immunodetect Nck1 protein in the cell lysates. NR1 levels were also detected in these samples (Sigma, 1:2000).

JNK-P Enzyme-linked immunosorbent assay (ELISA):-

25 μ g of protein isolated from the cortex of the *Nck1* -/- KO and WT mice was used in each ELISA well (PathScan Phospho-SAPK/JNK (Thr183/Tyr185) Sandwich ELISA kit, Cell Signalling). The assay will detect the levels of total phosphor-JNKs,

including p-JNK1, p-JNK2 and p-JNK3. 6 animals were used for each group. Three ELISA reactions were performed per sample, the mean of which were used in the data analysis. The JNK-P levels were statistically analysed using a two-tailed unpaired t-test.

SNP analysis

Human genomic DNA samples collected in the London area (London cohort) were derived from Prof. Hugh Gurling (UCL, UK) (approximately 300 control + 300 schizophrenic gDNAs, London cohort). These samples had been tested previously for stratification (Curtis et al., 1994). In addition, 392 gDNAs had been derived from anonymous donor blood samples from the West of Scotland (Glasgow cohort).

PCR –based assays were designed for NCBI SNP rs4804833. The primers were Fr4 (GTCGGTTCCTAGCCATCTCTGCAG) with Rr4 (GGTGCAGATGTCCAAGCAGCGATG) for rs4804833, yielding a 230bp product. Fr4/Rr4 could amplify PCR fragments that could/could not be cleaved with BsiEI at the site of the SNP, depending on the alleles present. Therefore, the second assay for *MAP2K7* was by RFLP using Fr4/Rr4 in conjunction with BsiEI.

Purification of recombinant human PPM1E and PPM1F

Human PPM1E cDNA (GenBank Accession number NM_014906) was inserted into pET30a to generate His-PPM1E, which encodes full-length PPM1E with an N-terminal His-tag.

This construct was transformed into *E. coli* strain BL21(DE3). The transformed bacteria were grown in LB medium, and then isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After

recombinant proteins were sufficiently induced, the bacteria were harvested by centrifugation and the harvested cell pellets were suspended in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS), and then disrupted by sonication. The extract was cleared by centrifugation, and the supernatants were used for the purification of recombinant proteins using Talon Co²⁺ column chromatography according to the manufacturer's instruction (Clontech). All the purification procedures were carried out at 4°C. His-PPM1E fractions eluted from the column stepwise with TBS containing 150 mM and 200 mM imidazole (Figure 8A). Similarly recombinant human PPM1F also purified from *E. coli* transformed with pET30a containing human PPM1F cDNA (GenBank Accession number NM_014634) (Figure 8B). Protein concentrations were determined by the method of Bradford with BSA as a standard.

CaMKIV Phosphatases Assay Development

Four kinds of assay development, BIOMOL GREEN, α Screen and HTRF for the measurement of purified human recombinant PPM1E phosphatase activity were conducted. Synthesized partial CaMKIV peptide (184-204), biotinylated-LSKIVEHQVLMKT(p)VCGTPGYC, as a substrate of PPM1E was applied for enzymatic reaction in assay development for BIOMOL GREEN, α Screen and HTRF.

BIOMOL GREEN

BIOMOL GREEN reagent was purchased from BIOMOL International. Phosphatase assay was carried out at RT for 1hr using purified PPM1E and biotinylated pThr-CaMKIV peptide. The reaction was stopped by adding BIOMOL GREEN reagent to capture the released Pi from the peptide. The mixture was allowed to stand at RT for 20 min, then the absorbance at 620 nm was measured. Increase of

the value at 620 nm means increase of Pi released from biotinylated pThr-CaMKIV peptide by phosphatase activity of PPM1E (Figure 9).

AlphaScreen Phosphosensor

AlphaScreen Phosphosensor kit was purchased from PerkinElmer. Phosphatase assay was carried out at RT for 1hr using purified PPM1E and biotinylated pThr-CaMKIV peptide. According to manufacturer' instruction (PerkinElmer), the mixture in which streptavidin donor beads and phosphosensor acceptor beads were added to capture biotinylated pThr-CaMKIV peptide allowed to stand at RT for 1hr in the dark room. After incubation α signal was measured by Fusion α HT (PerkinElmer). Decrease of the value of α signal means decrease of pThr-CaMKIV peptide by phosphatase activity of PPM1E (Figure 10).

HTRF: Homogenous Time Resolved Fluorescence assay

Streptavidin-XL^{entl} and Anti-phospho Threonine Polyclonal antibody cryptate were purchased from CIS bio International. Phosphatase assay was carried out at RT for 1hr using purified PPM1E and biotinylated pThr-CaMKIV peptide. According to manufacturer' instruction (CIS bio International), the mixture in which Streptavidin-XL^{entl} and Anti-phosphoThreonine Polyclonal antibody cryptate were added to capture biotinylated pThr-CaMKIV peptide allowed to stand at 4°C overnight. After incubation FRET was measured by Analyst GT (Molecular Devices). Decrease of the FRET value means decrease of pThr-CaMKIV peptide by phosphatase activity of PPM1E (Figure 11).

Semi-quantitative 2-deoxyglucose autoradiography:-

Adult *Nckl* $-/-$ KO and ICR $+/+$ wild-type control mice were used for this study. At 5 minute intervals, a mouse was placed in a cylindrical restrainer and its tail immersed in warm water (to aid visualization of the tail veins) for 5 mins. A bolus dose of [14-C]-2-deoxyglucose ([14-C]-2-DG; 120 μ Ci/Kg) was then administered via the lateral tail vein. The mouse was then placed in a normal home cage. 45mins later the mouse was replaced in the cylindrical restrainer and killed by an overdose of pentobarbitone (Sagittal, 42mg/kg) via the lateral tail vein. The brain was removed and frozen at -42C in isopentane cooled by dry ice and then stored at -70C. The brain was sectioned at -20C on a cryostat (Leica), with 3 adjacent 20 μ m sections being collected onto coverslips simultaneously every 200 μ m throughout the brain. The sections were immediately dried on a hotplate at 60C for 5mins to prevent diffusion of the isotope. The sections were then glued to cardboard and exposed to x-ray film (Biomax MR1, Kodak) with [14-C]-methylmethacrylate tissue standards for 2 days. Films were analysed using the MCID 5 computer based densitometry system. Tissue concentrations of [14-C]-2-DG were measured in discrete brain regions and 2-DG uptake calculated as follows:

[14-C]-2-DG (nCi/g) in region of interest

[14-C]-2-DG (nCi/g) in whole brain

Independent t-tests were carried out using SPSS software (v11) followed by application of the sequential Bonferroni correction method to correct for multiple comparisons. Levene's test of variance was used to confirm the homogeneity of the data.

Results and Discussion

Little is known about the genes that cause schizophrenia, or more specifically any alteration of expression/mutation of genes in a patient suffering from schizophrenia. Experiments were therefore designed to identify genes potentially contributing to the aetiology of schizophrenia, using prefrontal cortex tissue from rats treated according to the model of Cochran et al (2003), and post-mortem prefrontal cortex tissue from patients with schizophrenia, and controls.

A microarray screen was conducted, monitoring the expression of around 20,000 transcripts – theoretically the majority of the transcriptome – in prefrontal cortex of rats treated chronically with PCP according to our model (Cochran et al., 2003), as compared to rats treated chronically with vehicle. In this study, 327 transcripts were detected as significantly differentially-expressed in the prefrontal cortex after PCP treatment, as compared to vehicle treatment.

A parallel comprehensive microarray screen was then conducted, monitoring the expression of around 20,000 transcripts in post-mortem prefrontal cortex tissue (Brodmann area 10) from schizophrenia patients, as compared to equivalent tissue from control subjects. The subjects were matched for age, gender and post-mortem delay. In this study, 961 transcripts were detected as significantly differentially-expressed in the prefrontal cortex in schizophrenic patients, as compared to control subjects.

Previous microarray studies using tissue from post-mortem tissue from schizophrenic patients have identified altered expression of metabolic genes (Middleton et al., 2002), lipid-regulatory genes (Mimmack et al., 2002), presynaptic vesicle release genes (Mirnics et al., 2000) or myelin-related genes (Hakak et al., 2001). The present study, which was conducted with the most rigorous experimental

and analytical approaches, failed to confirm these findings. The investigators searched for patterns of functionally-related genes in the results from microarray studies. Based on considerable knowledge of cell biology and neuroscience, built on many years of experience, it was noted that a number of the genes identified in both the rat and human studies could potentially be linked together into a single biochemical signalling pathway. The investigators assessed the list of differentially-expressed genes in the rat study, and noted the presence of 4 protein kinases that could potentially be involved in a single signalling pathway downstream of NMDA receptors, together with a binding protein known to interact with one of these kinases (Table 1). The investigators have assessed the list of differentially-expressed genes in their human study, and noted the presence of these 4 protein kinases, together with a number of other genes encoding proteins potentially interacting with these kinases (Table 2).

There are a number of distinct mitogen-activated protein kinase (MAP kinase) signalling cascades in cells, in which a number of protein kinase enzymes act sequentially "in series" to phosphorylate and activate their downstream targets. In each case, a triggering signal phosphorylates and activates a MAP4K, which then leads to sequential phosphorylation of a MAP3K, a MAP2K, and a MAPK. In the many hundreds of genes identified in their screens, the investigators noted the presence of a set of genes that are likely to be functionally linked to each other. These genes, with their synonyms, are listed in Table 3.

MAPK9 (also known as JNK2), for example, is phosphorylated and activated by MAP2K7 (also known as MKK7). The activation of MAPK9 by MAP2K7 is relatively selective (Fleming et al., 2000; Machida et al., 2004). MAP2K7 in turn is phosphorylated and activated by MAP3K7 (also known as TAK1), which itself is

phosphorylated and activated by MAP4K4 (also known as HPK/GCK-like kinase – HGK, or Nck-interacting kinase – NIK) (Yao et al., 1999). The action of MAP kinase signalling cascades is co-ordinated by, and is dependent upon, specific scaffolding molecules which bring the kinases together. MAP4K4 interacts with the scaffolding protein Nck1, leading specifically to the activation of MAPK9 (Machida et al., 2004). Another MAP4K – Traf2 and Nck-interacting kinase (TNIK, or KIAA0551) also reportedly interacts with Nck and activates JNKs such as MAPK9 (Fu et al., 1999). In addition to MAP4K4 and TNIK, Nck1 also binds other scaffolding molecules such as GRB-associated proteins 1 & 2 (Gab1 & Gab2) and Crk-like protein (CrkL). CrkL over-expression can induce activation of JNKs such as MAPK9 (Ling et al., 1999). Nck1 over-expression in non-neuronal cells increases the activity of MAPK9 (JNK2) but not MAPK8 (JNK1) (Mizuno et al., 2002) suggesting that Nck1 function, like that of MAP2K7, is specifically linked to MAPK9 activation. Nck1 over-expression also alters the activity of another kinase PAK1 (Poitras et al., 2003), and PAK1 has been implicated in the regulation of JNK activity (Brown et al., 1996). Thus Nck1 can mediate parallel activation of PAK1 and JNKs (Schmitz et al., 2001).

PTK2B (protein tyrosine kinase 2B, also known as PYK2) has also been reported to mediate activation of JNKs (c-jun N-terminal kinases) such as MAPK9 (Blaukat et al., 1999). The small GTPase regulatory protein RICS (also known as GRIT or GC-GAP) binds to CrkL (Nakamura et al., 2002), Gab1 and Gab2 (Zhao et al., 2003) and can be activated by NMDA receptor stimulation (Nakazawa et al., 2003; Okabe et al., 2003). Hence NMDA receptor – dependent signalling can be predicted to propagate to Gab1 and Gab2. Similarly, CamKIV is activated by NMDA receptor stimulation via CamKK, and potentiates activation of JNKs such as MAPK9 (Enslen et al., 1996). PPM1E and PPM1F (fem-2) have recently been identified as

phosphatases that suppress the activity of both PAK1 (Koh et al., 2002) and CamK4 (Tan et al., 2001), and hence are intimately linked to the other proteins in this pathway. Indeed the direct interaction of CamKIV with PPM1E and PPM1F in neuronal cells was confirmed (see Figure 7).

These 16 genes can therefore be placed on a single biochemical signalling pathway, which the investigators have termed the Pyk-Nck-Jnk (for Pyk2-Nck1-Jnk2) pathway (Figure 1). Hence the investigators data suggested that the expression of a number of genes with potentially a very close functional interaction might be dysregulated in parallel in prefrontal cortex tissue from schizophrenic patients. The fact that there was an overlap between the changes in expression of Pyk-Nck-Jnk genes observed in the rat chronic PCP model of schizophrenia, and in the post-mortem tissue from patients, is consistent with the hypothesis that these changes in gene expression are causally involved in producing the core neurobiological deficits of schizophrenia (for example the metabolic hypo-frontality or the compromised chandelier cell function) or indeed the behavioural impairments such as reduced attention and impaired executive function.

To confirm the changes in the expression of these genes identified in the microarray studies, the investigators used real-time quantitative reverse-transcriptase PCR (RT-qPCR) to determine the level of expression of these genes accurately. Many of the genes were detected as differentially expressed in prefrontal cortex tissue from rats treated chronically with PCP, or from schizophrenic patients, relative to corresponding control tissue (Figure 2). These results reveal that in schizophrenia there is a co-ordinated dysregulation of this interacting set of genes.

MAPK9 has also been functionally linked to NMDA signalling, and in particular to the long-term effects of NMDA receptor stimulation on network activity

in the hippocampus and cortex (Chen et al., 2005). Hence one of the predicted consequences of Pyk-Nck-Jnk hypofunction in schizophrenia (as indicated by the present) is reduced NMDA receptor-dependent network activity.

Since Nck1 potentially has a central role in co-ordinating the various proteins of the Pyk-Nck-Jnk pathway, the investigators tested the hypothesis that manipulating the level of Nck1 expression in cultured neuronal cells would alter the level of JNK activity. It was found that over-expression of exogenous Nck1 in the neurones elevated JNK activity, as assessed by a phosphor-JNK specific ELISA (Figure 3).

Without wishing to be bound by theory, if the Pyk-Nck-Jnk pathway plays a major role in the aetiology of schizophrenia, then mice with altered Pyk-Nck-Jnk activity should show some of the neurochemical and metabolic changes characteristic of the disease. Mice were obtained with a targeted deletion of the Nck1 gene (Nck1 knockout mice) from the laboratory of Prof. T. Pawson, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto (Bladt et al., 2003). Relative to control mice, these mice seemed overtly normal. However, when cortex tissue was investigated from Nck1 knockout mice, it was found that the levels of the NMDA receptor NR1 subunit were elevated, as has been observed in the cortex of patients with schizophrenia (Dracheva et al., 2001; Ishimaru et al., 1994). Strikingly, the levels of parvalbumin mRNA were decreased in the prefrontal cortex in mice lacking Nck1 (and hence lacking a functional Pyk-Nck-Jnk pathway) relative to normal mice (Figure 4a). The decrease in parvalbumin expression is highly characteristic of the changes observed in post-mortem tissue from patients with schizophrenia (Beasley and Reynolds, 1997) suggesting strongly that reduced Pyk-Nck-Jnk pathway activity may be the direct cause of this pathology.

Schizophrenia patients show a highly characteristic pattern of altered metabolic activity in the CNS, as assessed by de-oxyglucose imaging or functional MRI. This pattern involves decreased activity in the prefrontal cortex relative to normal subjects (hypofrontality) and increased activity in parts of the auditory system during hallucinations (Tamminga et al., 1992; Wolkin et al., 1992; Shergill *et al.*, 2000). As a final test of the potential of Pyk-Nck-Jnk pathway dysfunction to cause the symptoms of schizophrenia, we assessed metabolic activity in the CNS of mice with a deleted Nck1 gene, relative to controls, by de-oxyglucose imaging. We found a pattern of altered metabolic activity that matched that observed in patients with schizophrenia, encompassing hypofrontality (Figure 4b) and increased auditory system activity (Figure 4c).

The existence of a close relative with schizophrenia is known to considerably increase the risk of someone suffering from schizophrenia, and hence schizophrenia is regarded as having a substantial genetic component. A number of different chromosomal loci have been linked with schizophrenia, although none of them particularly strongly. This has led to the idea that schizophrenia is a polygenic disease – with a number of different genes at different chromosomal loci all contributing to an increased risk of inheriting the disease. This raises the possibility that these different genes might all share a functional relationship, and hence a slight inherited functional impairment in a number of these genes might summate to give rise to the disease. The present data linking the Pyk-Nck-Jnk pathway genes to schizophrenia provides a rational basis for the contribution of a number of different genes to the aetiology of a single disease.

If dysfunction of the Pyk-Nck-Jnk pathway is a major factor in causing schizophrenia, it should be possible to obtain evidence of genetic linkage of a Pyk-

Nck-Jnk pathway gene to schizophrenia. The investigators selected MAP2K7, as a gene with a central role in Pyk-Nck-Jnk pathway function, and assessed the prevalence of single nucleotide polymorphisms (SNPs) in DNA from schizophrenia patients and control subjects. Two sets of blood sample DNA were analysed, one collected in the Greater London area and comprising around 300 patients and 300 controls, and the other collected in the Glasgow/West of Scotland area, comprising around 200 patients and 200 controls. Any significant effects that are reproduced in both of the two separate sets of samples are likely to be highly relevant to the causes of schizophrenia.

The results of SNP analysis for MAP2K7 in human blood samples from schizophrenic patients and controls (Figure 5) showed that for the 1st SNP, there was a significant association with schizophrenia in the London samples, that was replicated in the separate Glasgow samples (Figure 5). Furthermore, for the 2nd SNP, there was a similar significant association with schizophrenia in the London samples, that was replicated in the separate Glasgow samples (Figure 5). When the two sets of samples were analysed together, a very high level of significance for the association with schizophrenia was noted for both SNP1 and SNP2 (Figure 6). Thus a highly significant association of MAP2K7 SNPs with schizophrenia is detected, using two different SNPs, and two different sets of samples.

Thus the investigators have observed:

- 1) altered expression, in prefrontal cortex tissue from schizophrenia patients, of a number of functionally-related genes that constitute a single signalling pathway in the brain;
- 2) altered parvalbumin cell function in mice with genetically-modified activity of this signalling pathway; and

3) metabolic hypofrontality and elevated auditory system metabolic activity – a pattern highly characteristic of the brains of patients with schizophrenia – in mice with genetically-modified activity of this signalling pathway; and

4) highly significant genetic linkage between a key gene in this signalling pathway and schizophrenia.

These data imply that dysfunction of this Pyk-Nck-Jnk pathway, caused by impaired function of any individual gene in the pathway, can be a cause of schizophrenia, and hence that novel drug treatments, aimed at restoring the function of this pathway, may be a highly effective strategy for curing the disease.

Table 1: Relative changes in expression of Pyk-Nck-Jnk pathway genes, in prefrontal cortex, in rats treated chronically with PCP, relative to corresponding controls, as assessed by microarray analysis.

Gene	% change in rat prefrontal cortex after chronic PCP
PTK2B / PYK2	16% down
CAMK4	23% down
TNIK	16% down
MAPK9	27% down
MAPK8ip1	20% down

Table 2: Relative changes in expression of Pyk-Nck-Jnk pathway genes, in prefrontal cortex, in schizophrenic patients, relative to corresponding controls, as assessed by microarray analysis.

Gene	% change in prefrontal cortex from schizophrenic patients
RICS / GRIT	12% down
PTK2B / PYK2	15% down
Gab1	19% up
Gab2	20% up
Nck1	15% down
CrkL	14% down
CamKK	12% down
CAMK4	33% down
MAP4K4	18% up
TNIK	22% down
MAP3K7	14% down
MAPK9	23% down

Table 3:

Most common gene name	Abbreviation (s)	mRNA/ protein Acc.No.	Synonyms
GTPase interacting with TrkA	GRIT / RICS	NM_014715 NP_055530	GC-GAP, KIAA0712, MGC1892, RICS, p200RhoGAP, p250GAP
Protein tyrosine kinase 2 beta,	PTK2B / PYK2	NM_004103 NP_004094	Focal adhesion kinase 2 (FAK2 / FADK 2), Cell adhesion kinase beta (CAK beta), Calcium-dependent tyrosine kinase (CADTK), Related adhesion focal tyrosine kinase (RAFTK)
GRB2-associated binding protein 1	Gab1	NM_002039 NP_002030	
GRB2-associated binding protein 2	Gab2	NM_012296 NP_036428	KIAA0571
NCK adaptor protein 1	Nck1	NM_006153 NP_006144	NCKalpha
NCK-associated protein 1	NckAP1	NM_013436 NP_038464	Hem-2, NAP-1, NAP125
Crk-like protein	CrkL	NM_005207 NP_005198	v-crk avian sarcoma virus CT10 oncogene homolog-like
p21-activated protein kinase	PAK1	NM_002576 NP_002567	PAKalpha
protein phosphatase 1E	PPM1E	NM_014906 NP_055721	Partner of PIX 1 (POPX1), KIAA1072 Protein phosphatase 2C eta,
protein phosphatase 1F	PPM1F	NM_014634 NP_055449	Partner of PIX 2 (POPX2), hFEM-2 , Ca ²⁺ /calmodulin-dependent protein kinase phosphatase (CaM-kinase phosphatase, CaMKPase)
calcium/calmodulin-dependent protein kinase kinase 1, alpha	CamKK	NM_032294 NP_115670	CaMKK 1

calcium/calmodulin-dependent protein kinase IV	CAMK4 / CamKIV	NM_001744 NP_001735	CaMK-GR
mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	NM_004834 NP_004825	HPK/GCK-like kinase (HGK) Nck interacting kinase (NIK)
TRAF2 and NCK interacting kinase	TNIK / KIAA0551	NM_015028 NP_055843	KIAA0551
mitogen-activated protein kinase kinase kinase 7	MAP3K7	NM_003188 NP_003179	Transforming growth factor beta-activated kinase 1 (TAK1)
mitogen-activated protein kinase kinase 7	MKK7 / MAP2K7	NM_145185 NP_660186	JNK activating kinase 2, c-Jun N-terminal kinase kinase 2 (JNK kinase 2 / JNKK 2), MAPKK7
mitogen-activated protein kinase 9	MAPK9 / JNK2 / p54aSAPK	NM_002752 NP_002743	c-Jun N-terminal kinase 2, Stress-activated protein kinase JNK2, JNK2 alpha , JNK2 beta,
mitogen-activated protein kinase 8 interacting protein 1	MAPK8ip1	NM_005456 NP_005447	C-jun-amino-terminal kinase interacting protein 1 (JNK-interacting protein 1, JIP-1) Islet-brain-1 (IB-1)

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Claims

1. A method of prognosis or diagnosis of schizophrenia comprising the steps of:

- a) providing a tissue or body fluid sample from a test subject;
- b) detecting a level of expression or level of activity of one or more of the following genes/proteins PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1 in a cell or cells from said sample; and
- c) comparing the level of expression or level of activity of said one or more genes/proteins with a level of expression or level or activity of said one or more genes/proteins in a control sample from a subject not suffering or predisposed to suffering from schizophrenia.

2. A DNA array for use in a method according to the present invention the array comprising or consisting essentially of one or more of the following genes or sequence specific fragments thereof: PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1, the array being immobilised on a support.

3. A database comprising information regarding one or more genes selected from the group consisting of PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1, said information comprising details of expression levels that are characteristic of schizophrenia and non-

schizophrenia subjects, the database for use in the method according to any preceding claim.

4. The database according to claim 3 as stored in memory of a computer system or on a removable data medium.

5. A method of screening a compound for treating schizophrenia, which comprises:

(a) bringing a test compound into contact with a wild type cell or transformed cell which is capable of expressing one or more schizophrenia-associated genes selected from the group consisting of PPM1E; PPM1F; MAP2K7; MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1;

(b) detecting a level of expression or activity of said one or more schizophrenia-associated gene(s)/protein(s) in said cell; and

(c) selecting a compound which promotes or suppresses expression or activity of the schizophrenia-associated gene/protein in comparison with a control (vehicle).

6. A method for measuring an anti-schizophrenia effect of a compound using an animal model of schizophrenia, which comprises:

(a) administering the compound to an animal model displaying of schizophrenia characteristics;

(b) detecting a level of expression or activity of one or more genes/proteins selected from the group consisting of PPM1E; PPM1F; MAP2K7;

MAPK9; RICS/GRIT; PTK2B/PYK2; Gab1; Gab2; Nck1; NckAPI; CrkL; PAK1; CamKK; CAMK4; MAP4K4; TNIK; MAP3K7; and MAPK8ip1;

(c) comparing the level of expression or activity of said one or more genes/proteins with a level of expression or activity of said one or more genes/proteins in a further control animal displaying characteristics of schizophrenia, but which has not been administered the compound; and

(d) selecting compounds for further evaluation as potential anti-schizophrenia agents, which modulate expression or activity of said one or more genes/proteins, in comparison to the control animal.

7. The method according to either of claims 5 or 6 wherein the level of expression is detected by hybridisation studies, quantitative PCR, Northern blotting, Western blotting or an immunoassay.

8. The method according to either of claims 5 or 6 wherein the activity of the protein is detected by an enzyme assay or ligand/protein binding assay.

9. The method according to either of claims 5 or 6 wherein the protein is a kinase and its activity is detected by the use of antibodies or other specific binding agents which are capable of binding to a phosphorylated or non-phosphorylated form of the kinase's substrate.

10. The method according to any of claims 1 or 5 - 9 wherein at least 2, 3, 4, 5, 10 or 15 of the identified genes are included in said method.

11. The method according to any one of claims 1 to 5 wherein the genes/proteins to be tested are identified in Tables 1 or 2.

12. The method according to any one of claims 1 to 5 wherein the genes/proteins to be tested are one or more MAP kinases, such as MAP2K7, MAPK9, MAP3K7 and/or MAP4K4.

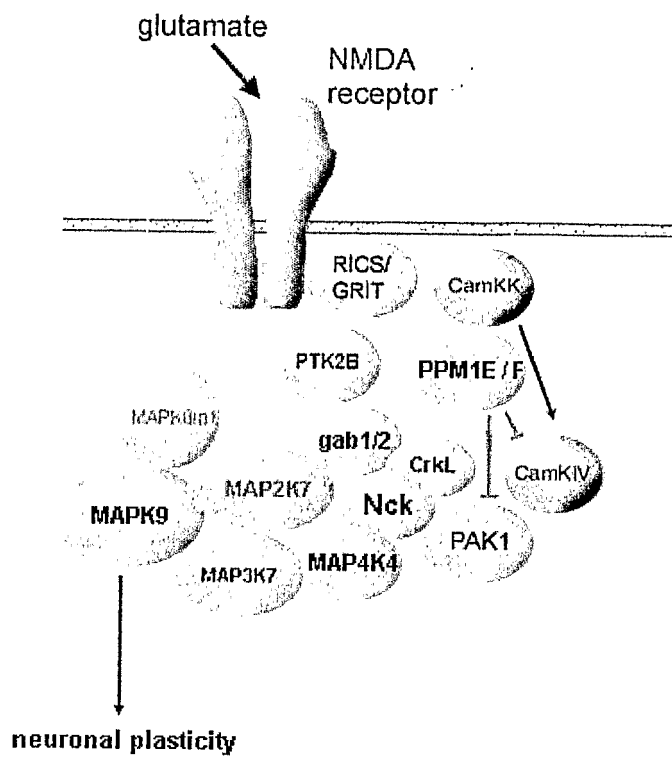
Figure 1:

Figure 2

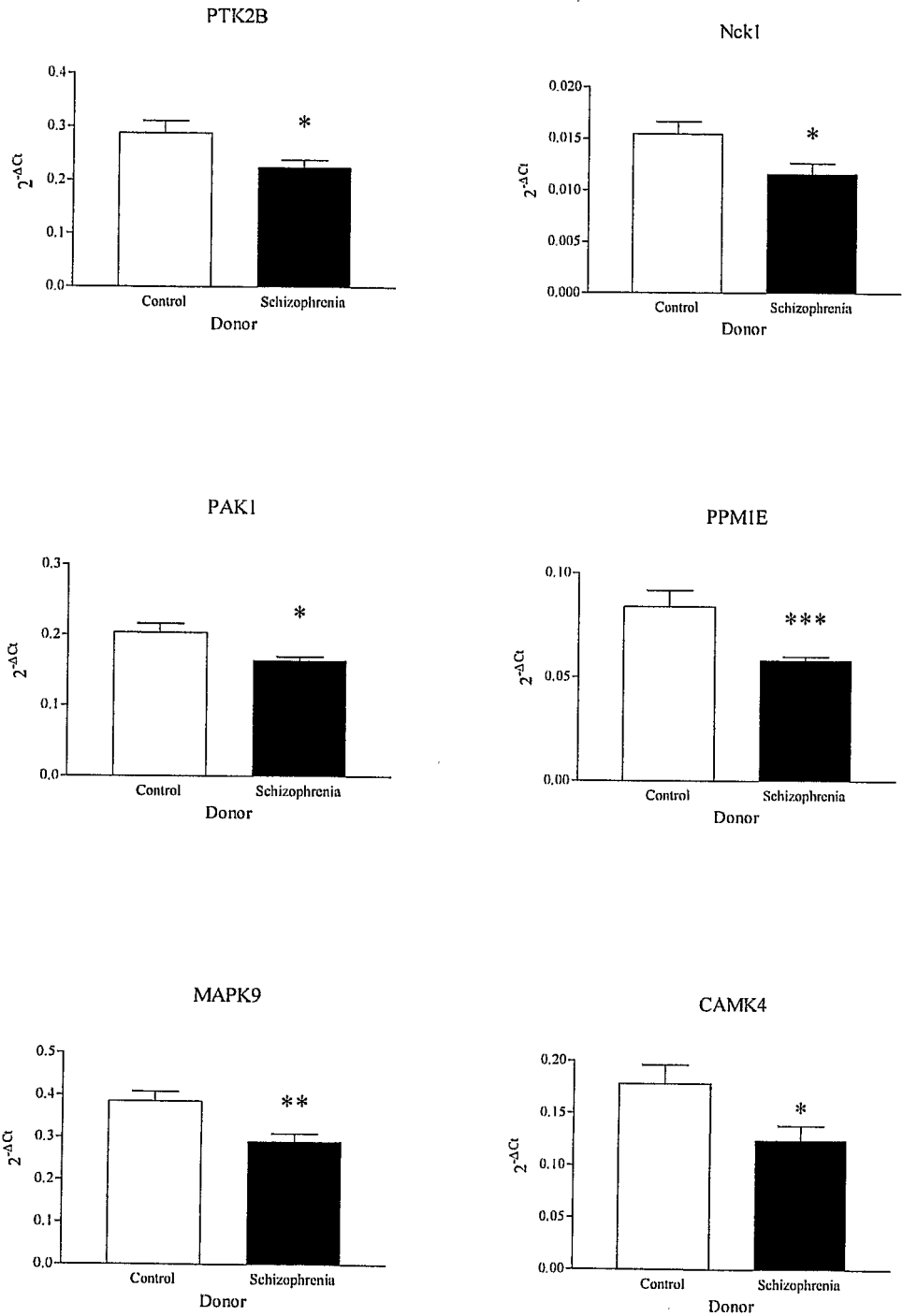


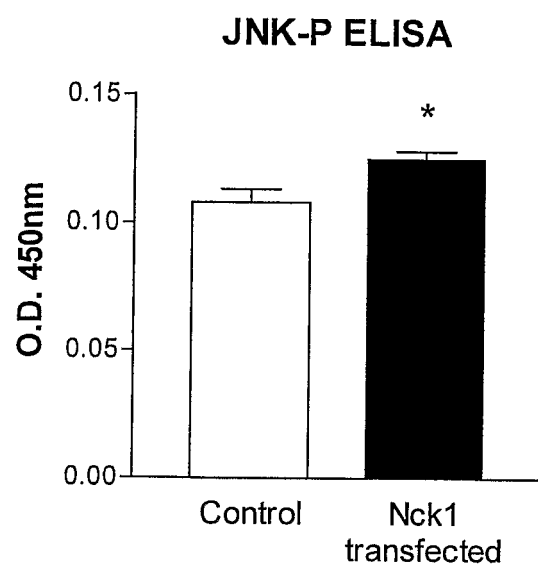
Figure 3:

Figure 4a:

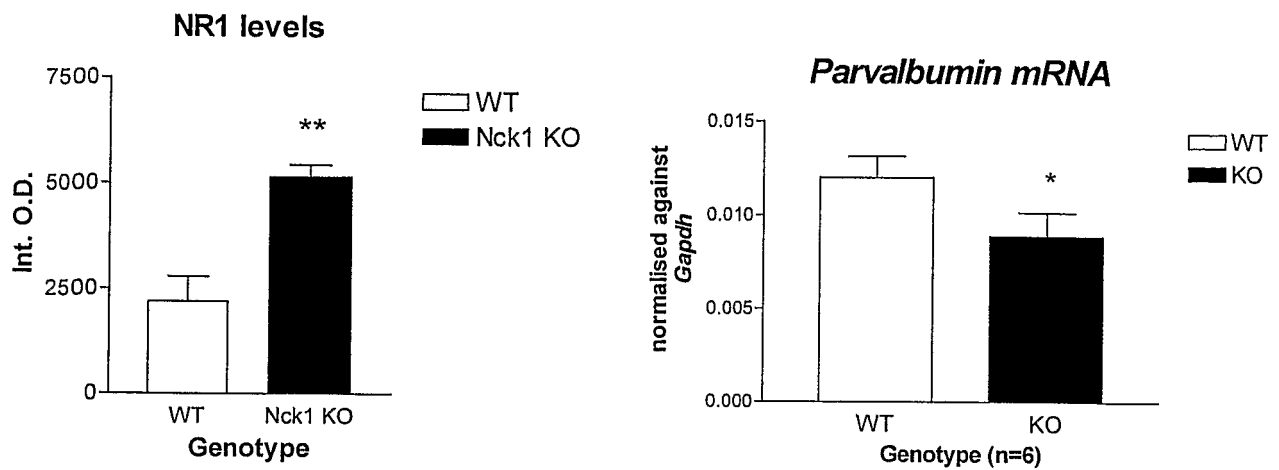


Figure 4b

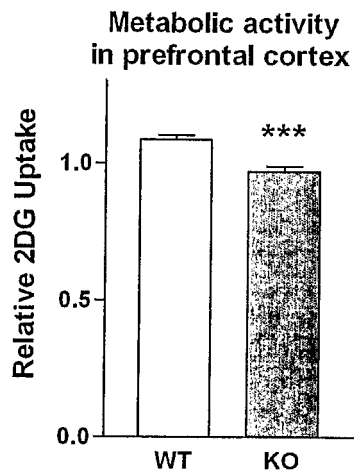


Figure 4c

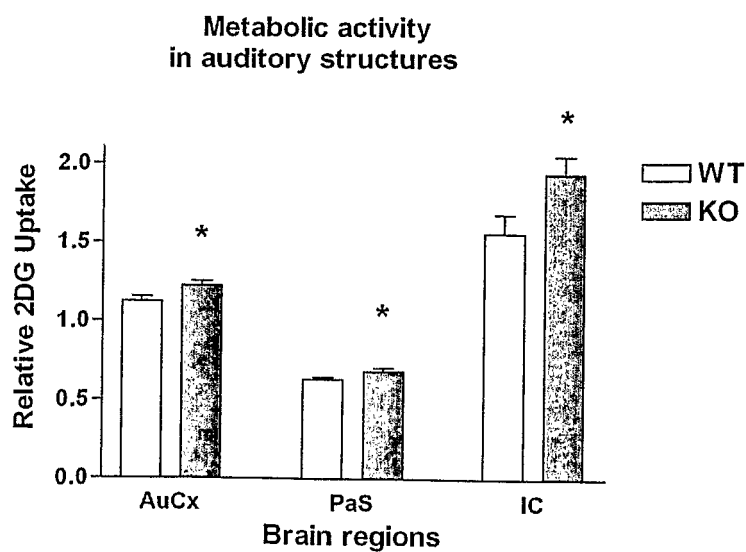


Figure 5:

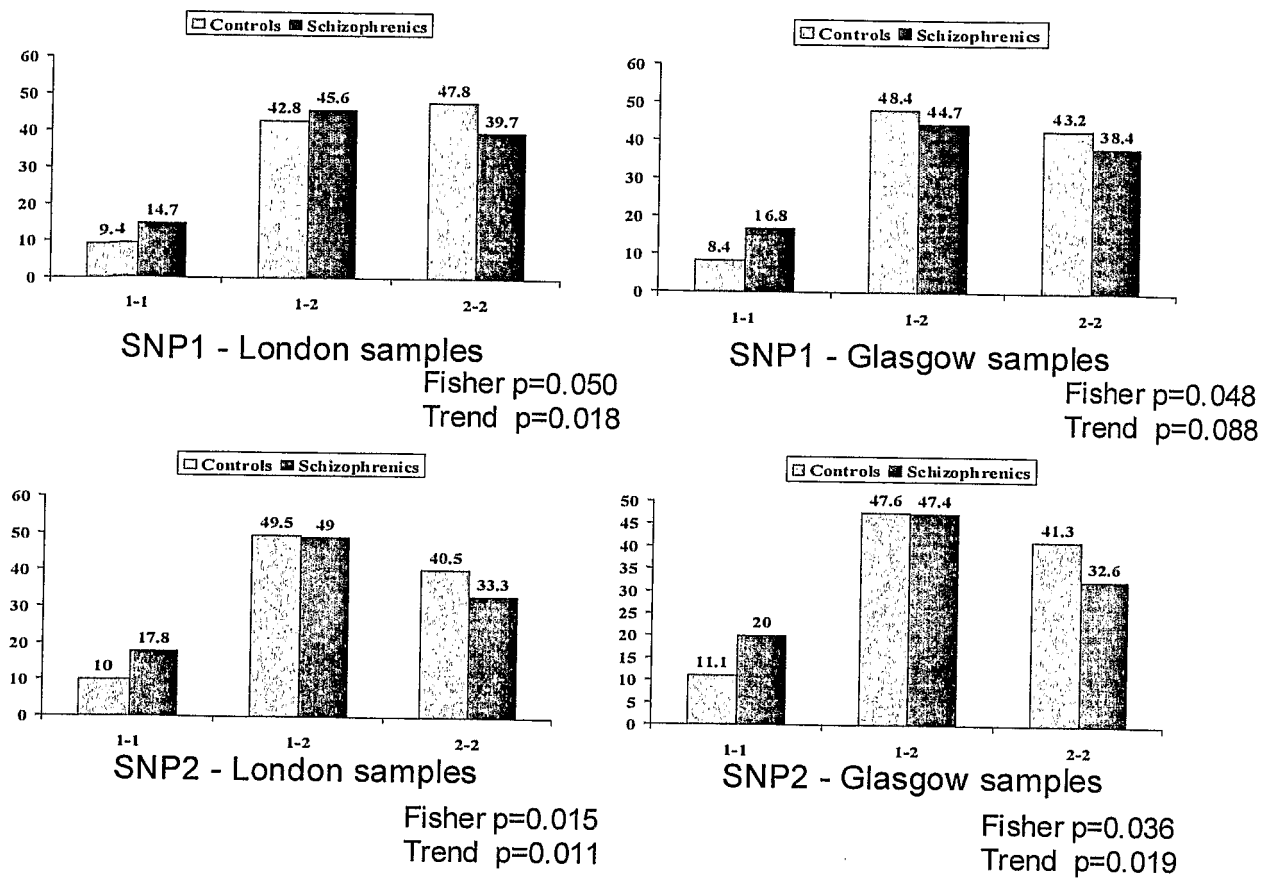
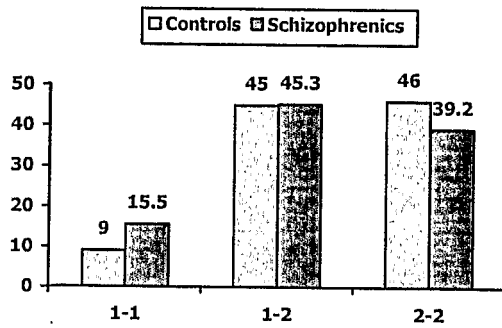
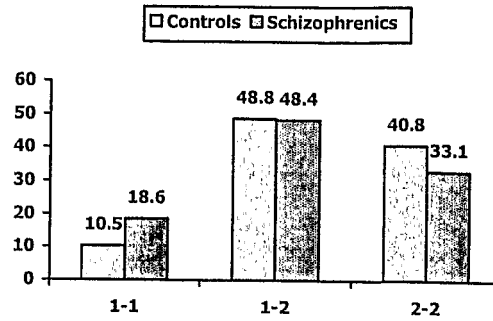


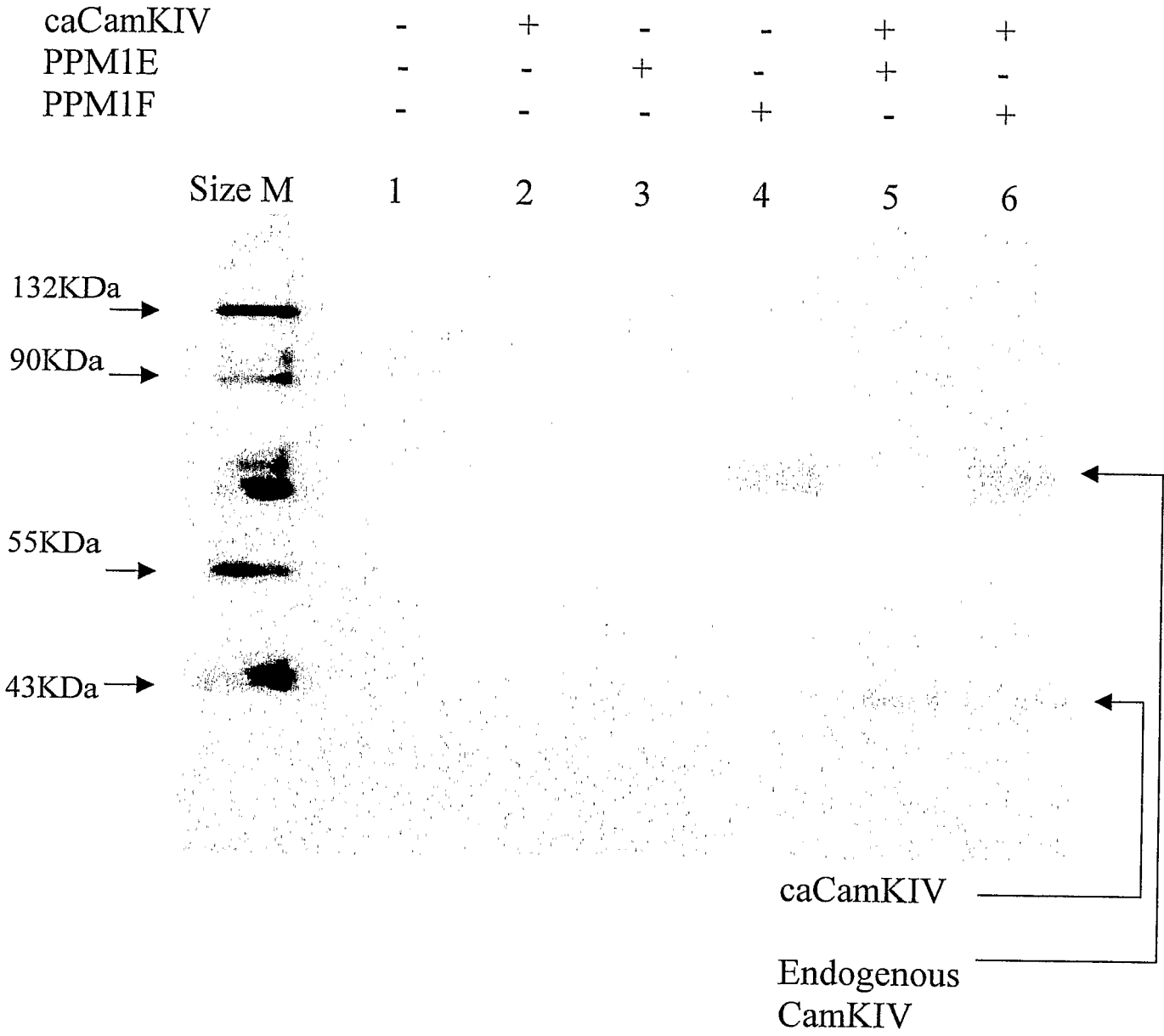
Figure 6:

SNP1 - Fisher $p=0.0037$
Trend $p=0.0039$



SNP2 - Fisher $p=0.0005$
Trend $p=0.0005$

Figure 7:



Figures 8a and 8b

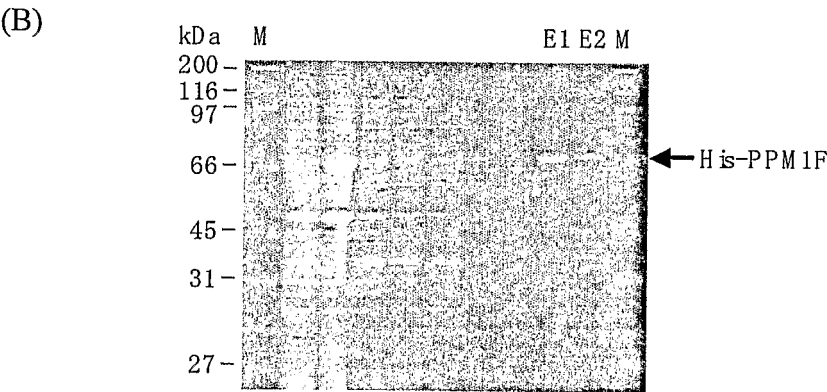
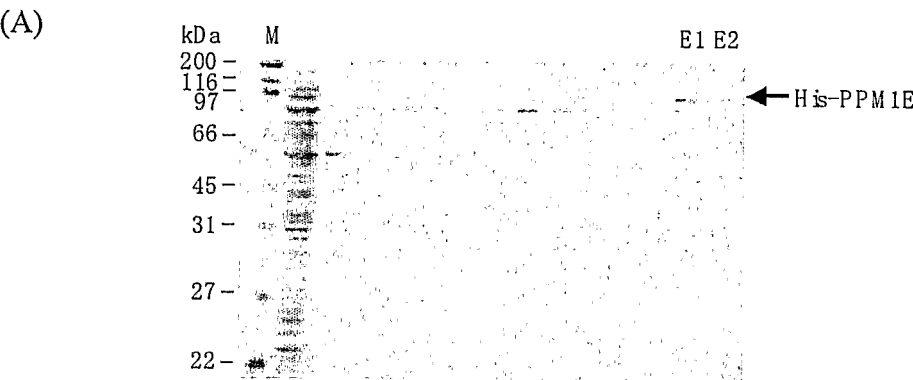


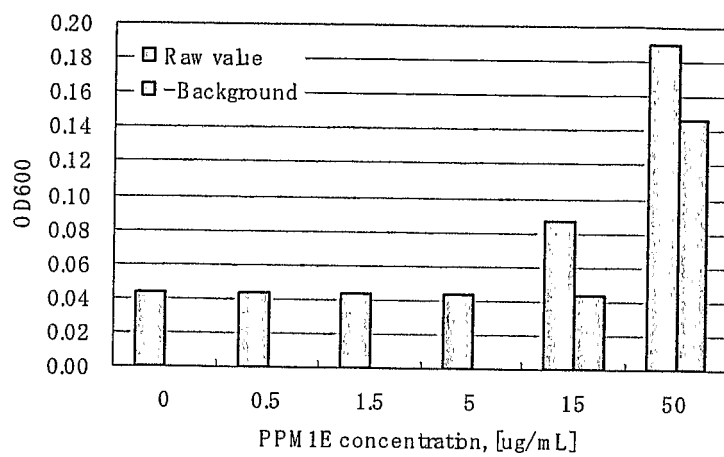
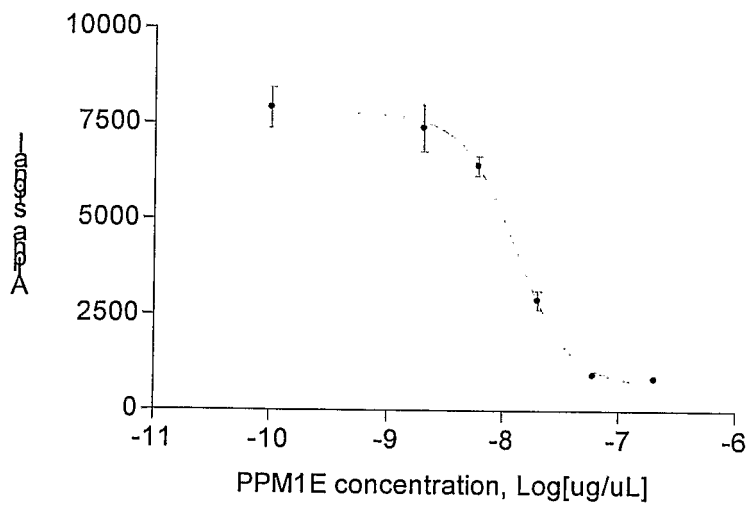
Figure 9**Figure 10**

Figure 11

