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ORIGINAL RESEARCH ARTICLE

Comparative proteome analysis of the hippocampus implicates chromosome 6q in schizophrenia

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Comparative brain proteome analysis is a new strategy to discover proteins and therefore genes whose altered expression may underlie schizophrenia. This strategy does not require an *a priori* theory of the pathogenesis or the mode of inheritance of schizophrenia. Using proteome analysis we previously compared the hippocampal proteome, that is, those proteins expressed by the hippocampal genome, of seven schizophrenic individuals with the hippocampal proteome of seven control individuals, matched for age and post mortem delay.[1] We found 18 proteins that were significantly altered in concentration in the schizophrenic hippocampus ($P < 0.05$), when compared to control tissue. One of these proteins was characterised, by N-terminal sequencing, as diazepam binding inhibitor whose gene maps to 6q12–q21. Here we characterise a further three of the 18 proteins as: manganese superoxide dismutase, 6q25.3, T-complex protein 1, 6q25.3–q26 and collapsin response mediator protein 2, 8p21. That three of these four characterised proteins should map to the long arm of the same chromosome is significant ($P < 0.002$) and suggests the importance of chromosome 6q in schizophrenia. These results indicate that antioxidant defence is altered in the schizophrenic hippocampus and suggest that segregation distortion, of schizophrenia susceptibility genes, may be a possible causative factor in the high incidence of schizophrenia. *Molecular Psychiatry* (2000) **5,** 85–90.

Recent advances in protein chemistry, in particular the advent of two-dimensional gel electrophoresis (2DE), coupled with ultra-micro protein sequencing and computer-assisted gel comparison, now enable the characterisation of a significant proportion of a proteome, that is, those proteins expressed in a given tissue.[2] Five to seven amino acids of protein sequence, isoelectric point (pI) and molecular mass (Mr) data can be derived from a single 2DE gel spot. This information is sufficient to characterise a protein, if it is represented in a sequence databank.[3] Changes in concentration of specific proteins in a tissue are also detectable by 2DE and reflect either altered expression of a gene, changes in protein turnover or post-translational modifications.[4] Therefore, 2DE has been used to detect proteins that are influenced by a disease process by comparing disease-affected tissue with unaffected tissue,[4] that is, by comparative proteome analysis.

No biochemical marker for schizophrenia has yet been documented. Although the organic basis for schizophrenia is unknown, cerebral ventricular enlargement, and therefore reduced brain tissue volume, stands as perhaps the most frequently confirmed neurobiological finding associated with schizophrenia.[5] The brain regions that consistently contribute to this reduction in volume include the hippocampal formation.[6] Therefore, we collected hippocampal tissue from the brains of seven schizophrenics and seven control individuals post mortem for comparative proteome analysis.[1] The mean age for the control group was 51 years, SD 17, and for the schizophrenic group it was 41 years. SD 13. The mean post mortem delay for the control group was 33 h, SD 15, and for the schizophrenic group it was 38 h, SD 11. Proteomes were visualised by 2DE of homogenised hippocampal tissue (Figure 1). To correct for variations in protein loading, normalisation between gels was carried out, by expressing features as a percentage of the sum of optical density for all features detected on the gel. Of a mean of 1845 ($n = 14$) spots detected per 2DE gel, it was possible to match 574 among the 14 samples.

The mean concentration of each matched protein in the schizophrenic hippocampal proteome ($n = 7$) was statistically compared with the mean concentration of the same protein in the control hippocampus ($n = 7$) using the *t*-test (two-tailed, heteroscedastic). In comparison with the control hippocampal proteome, nine proteins in the schizophrenic hippocampal proteome were significantly decreased and nine significantly increased in concentration ($P < 0.05$) (Figure 1).[1] These proteins were located on preparative Coomassie stained gels by alignment and matching to silver-stained analytical gels using the 2DE software Melanie II (Figure 2). It was possible to match 506 protein spots between Coomassie stained preparative gels and silver-stained analytical gels.

We previously characterised one of the proteins decreased in schizophrenia as diazepam binding inhibitor (DBI), by N-terminal sequencing of the tryptic digest as previously described.[1] Here we characterise a further three of these 18 proteins by the same methods (Figure 1, Table 1). Of the nine proteins that were decreased in concentration in the schizophrenic hippo-

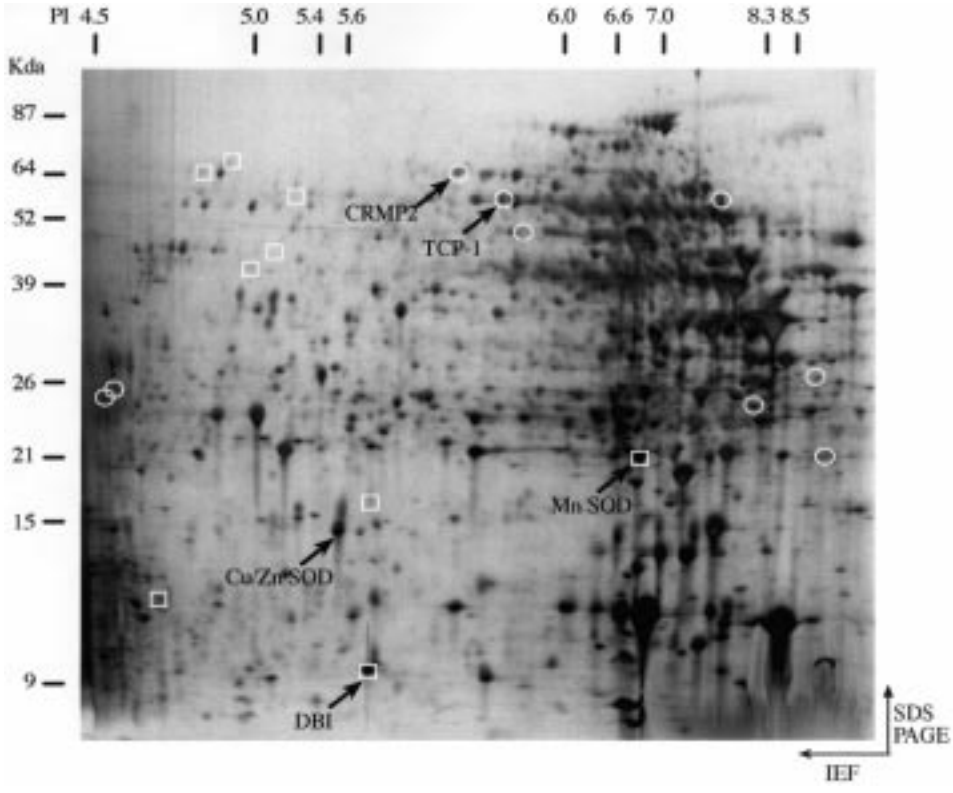

Figure 1 Silver-stained 2DE gel of human hippocampal tissue from a schizophrenic patient. Circles denote the nine proteins that were significantly increased in concentration ($P < 0.05$) and squares denote the nine proteins that were significantly decreased in concentration ($P < 0.05$) in the schizophrenic hippocampus, when compared to the control hippocampus.[1] The arrows and labels indicate proteins characterised by N-terminal protein sequencing. CRMP 2, collapsin response mediator protein; TCP-1, T-complex protein 1; Mn SOD, manganese superoxide dismutase; Cu/Zn SOD, copper zinc superoxide dismutase; DBI, diazepam binding inhibitor.

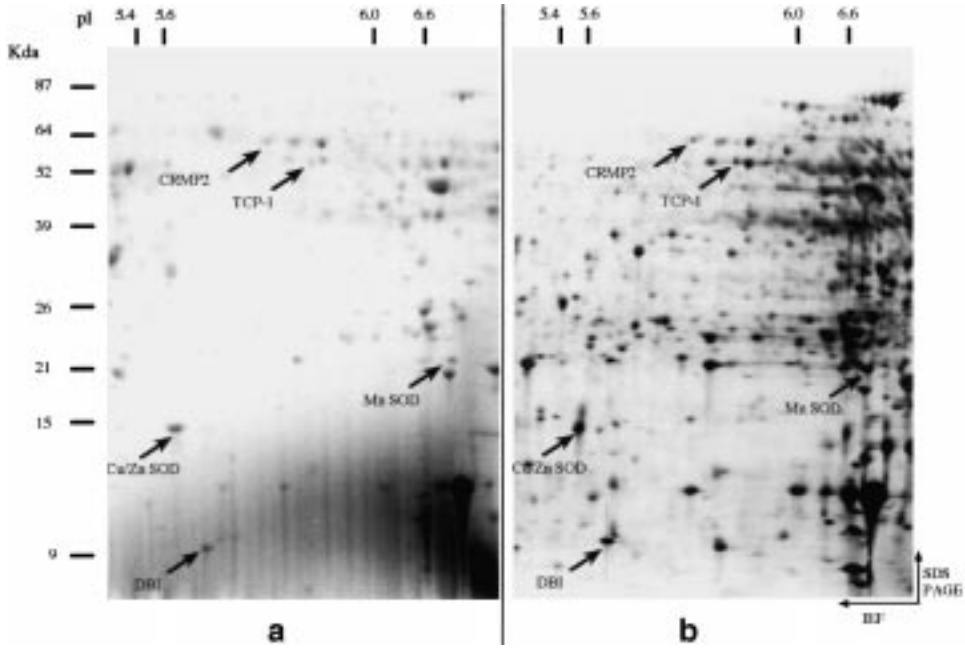

Figure 2 Alignment of a portion of a Coomassie-stained preparative 2DE gel of hippocampal tissue (a), from a schizophrenic individual, with a similar portion of a silver-stained analytical gel (b) from the same individual. Proteins indicated by arrows were identified previously[1] but collected and characterised here. Alignment and matching of gels was carried out using the analysis software Melanie II (see Methods).

Table 1 Proteins characterised from hippocampal schizophrenia tissue by 2DE and N-terminal sequencing

Peptide sequences	*Unique database match*	*Accession No.*	*Theoretical pI/Mr*	*Observed pI/Mr*	*Concentration*	*OMIM gene map location*	*Gene name*
HSLPDL, HHAAYV	Mn superoxide dismutase	P04179	6.86/22204[a]	6.7/21 000	decreased	6q25.3	SOD2
LLTHHK, AVAME	T-complex protein 1	AF026293	6.01/57 488	5.9/56 000	increased	6q25.3–q26	TCP1
MDENQFVA, SVIWDK, GTVV	Collapsin response mediator protein-2	Q16555	5.95/62 293	5.8/64 000	increased	8p21	CRMP2
VWGSIK	Cu/Zn superoxide dismutase	P00441	5.7/15 805	5.6/15 000	no change	21q22.1	SOD1

Theoretical pI and Mr were derived from database entries. Map locations were sourced from Online Mendelian Inheritance in Man, National Center for Biotechnology Information, www3.ncbi.nlm.nih.gov:80/Omim/
[a]Minus domain transit peptide.

campus, a second one was characterised and found to be manganese superoxide dismutase (Mn SOD, 6q25.3). Of the nine proteins that were increased in concentration, two were characterised and found to be T-complex protein 1 (TCP-1, 6q25.3–q26) and collapsin response mediator protein 2 (CRMP-2, 8p21). Both SOD2 and TCP1 were also significant ($P < 0.05$) using an unpaired non-parametric Mann–Whitney test. The fourteen remaining proteins were of lower abundance and remain to be characterised.

Mn SOD concentration was significantly decreased in the schizophrenic hippocampus. Therefore, Mn SOD may play a role in the pathogenesis of schizophrenia. Alternatively, Mn SOD could be altered in concentration by factors that are common to the environment of sufferers of schizophrenia. Mn SOD catalyses the dismutation of two molecules of peroxide radical to dioxygen and hydrogen peroxide in the mitochondria, providing antioxidant defence against lipid peroxidation. In the brain this protects the viability of neuronal membranes.[7] Altered SOD activity has been found in both the brain[8] and peripheral tissues[9] in schizophrenics and has been linked to the dementias of Downs syndrome and Alzheimer's disease.[10,11] Dementia is also common in elderly schizophrenics but is often attributed to their institutionalisation or medication.[12] In addition, acute occupational exposure to manganese, the metal ligand of Mn SOD, can cause schizophrenia-like symptoms.[13] Our findings suggest that antioxidant defence, via Mn SOD, is altered in the schizophrenic brain, however, it remains a possibility that this is a result of medication.

Among relatives of Ashkenazi schizophrenic probands, the rate of the neuromuscular disease amyotrophic lateral sclerosis (ALS) has been reported to be 3/1000, compared to expected population rates of approximately 2/100 000.[14] Mutations in the Cu/Zn superoxide dismutase gene (SOD1) cause both familial and sporadic forms of ALS. Although Cu/Zn SOD is structurally distinct from Mn SOD,[15] it is likely that mutations in the Mn SOD gene would cause similar symptoms to ALS.[7] Unfortunately, ALS in Ashkenazi schizophrenia pedigrees has not been characterised at the molecular level. We sought and identified Cu/Zn SOD on hippocampal 2DE and found it to be unchanged in concentration (Table 1, Figure 1). Therefore, the high rates of ALS in Ashkenazi schizophrenia pedigrees may indicate that the Mn SOD gene (SOD2, 6q25.3) is linked to schizophrenia susceptibility genes.

T-complex protein 1 (TCP-1), the second protein characterised, was increased in concentration in the schizophrenic hippocampus. TCP-1 is a molecular chaperone that plays a role, *in vitro*, in the folding of actin and tubulin, both of which are important members of the neuronal cytoskeleton.[16] Increased levels of TCP-1 could result from altered cytoskeletal turnover in neurons in the schizophrenic hippocampus.

Genetic susceptibility has been implicated in schizophrenia, and its persistence is puzzling in light of low fertility rates in affected individuals, especially males.[17] An animal model to explain the maintenance of deleterious genes was shown in mice. The *t* complex of genes, which confers cancer susceptibility, is transferred preferentially through fathers in a phenomenon called segregation distortion.[18] The *t* complex contains both TCP-1 and MnSOD, both of which were identified in this study. Figueroa *et al*[19] proposed that since Mn SOD is a mitochondrial enzyme, and mitochondria are important for sperm motility, MnSOD is in fact the gene within the *t* complex that is responsible for the observed segregation distortion. TCP-1, 6q25.3–q26, and Mn SOD, 6q25.3, also map together in humans. Therefore, altered Mn SOD levels in humans may cause segregation distortion and maintain linked schizophrenia susceptibility genes despite these genes also conferring reduced fertility.

The third protein characterised, collapsin response mediator protein 2 (CRMP-2, 8p21), was increased in concentration in the schizophrenic hippocampus. CRMP-2, also known as dihydropyrimidinase related protein-2, is homologous to *Caenorhabditis elegans* unc-33, which controls the guidance and outgrowth of neuronal axons initiated by collapsin.[20] CRMP-2 immunocytochemistry shows that CRMP-2 is exclus-

ively localised in the central and peripheral nervous systems in mouse embryos and is detectable in the adult brain at lower levels.[21] Our detection of increased levels of CRMP-2 in the schizophrenic hippocampus may, therefore, reflect an increased lability of neuronal interconnections. This lability is also found in early development.

DBI 6q12–q21, characterised in our previous study,[1] can down-regulate the action of gamma-aminobutyric acid (GABA) by binding to a benzodiazepine recognition site located on the $GABA_A$ receptor.[22] DBI may have a symptom modulatory role in schizophrenia.[23]

The four proteins described above were chosen for characterisation because they were amongst the most abundant of the original 18 identified. The probability of choosing by chance, four proteins from the hippocampal proteome, three of which map to the same arm of the same chromosome and the fourth to a different chromosome arm, is low, approximately 1 in 500 (see Methods). Therefore their co-location on 6q indicates this chromosomal region is important in schizophrenia. A susceptibility locus for schizophrenia exists between the loci for DBI and SODI/TCP-1 at 6q21–q22.3.[24] In this chromosomal region there are genes for two disorders, in addition to ALS, and two commonly elicited signs in sufferers, that co-segregate in some families with schizophrenia (Table 2). Therefore, heterogenous rearrangement of, deletions in, or abnormal regulation of genes within the chromosomal region 6q12–26 may account for many of the described features of familial schizophrenia and the changes in concentration of Mn SOD, DBI, and TCP-1 in the schizophrenic hippocampus.

In summary, 3% of the 2DE characterisable hippocampal proteome reflects the molecular events surrounding schizophrenia. The concentrations of DBI and Mn SOD are significantly reduced in the schizophrenic hippocampus whereas TCP-1 and CRMP-2 are significantly increased. A speculative scenario is that susceptibility to damage from reactive oxygen species is greater in the schizophrenic hippocampus resulting in an increased turnover of neuronal interconnections. The high frequency of the superoxide dismutase disorder, ALS, in Ashkenazi schizophrenic pedigrees and the co-location of the genes for Mn SOD, TCP-1 and DBI to chromosome 6q suggests that this scenario is due to shared instability in the chromosomal region 6q12–26 rather than shared environmental influences. Therefore, these proteins may prove useful as markers for susceptibility to schizophrenia. In addition, the proposal that Mn SOD may cause segregation distortion suggests a possible mechanism for the maintenance of schizophrenia susceptibility genes in the population despite these genes conferring low fertility.

Table 2 Genes located between the loci for DBI and SOD2/TCP1 on chromosome 6q relevant to schizophrenia

OMIM gene name	*Locus*	*Status*	*Symptoms, signs, disorders in schizophrenia*	*References*
DBI	6q12–q21	I	Sleep REM latency correlated with haloperidol treatment, paranoia	23
HTR1B	6q13	P	Hallucinations, anhedonia	28
HTR1E	6q14–q15	P	Hallucinations, anhedonia	28
CNR1	6q14–q15	P	A cannabinoid receptor experimentally linked to **reduced amplitude P300 brain potentials**	27
RP24	6q14–q21	P	**Retinitis pigmentosa disorder** co-segregates in schizophrenia pedigrees	14,26,29
GABRR1 and 2	6q14–q21	P	Expressed primarily in the retina and control lateral (**saccadic**) eye movements	29
DFNA10	6q22.2–q23.3	C	**Sensorineural deafness disorder** co-segregates in schizophrenia pedigrees	15,26
ESR1	6q25.1	C	Differences in the age of onset and symptoms of schizophrenia in women compared with men implicates oestrogen	30
SOD2	6q25.3	C	**ALS**, neuronal degeneration, disorder co-segregates in schizophrenia pedigrees, segregation distortion	7–15,17–19
TCP1	6q25.3–q26	C	Cytoskeletal maintenance, neurotransmitter trafficking, segregation distortion	16–19

Genes in bold were identified by 2DE. Signs and disorders in bold are those referred to in the main text. Map locations were sourced from Online Mendelian Inheritance in Man, National Center for Biotechnology Information, www3.ncbi.nlm.nih.gov:80/Omim/

Gene location status: I = inconsistent, P = provisional, C = confirmed.

DBI, Diazepam-binding inhibitor; HTR1B, and -IE 5-hydroxytryptamine (serotonin) receptor-1B, and 1E; CNR1, cannabinoid receptor-1; RP24, retinitis pigmentosa-24; GABRR1, and 2, gamma-aminobutyric acid (GABA) A receptor, rho-1 and 2; DFNA10, deafness, autosomal nonsyndromic sensorineural, brain; ESR1, oestrogen receptor 1; SOD2, superoxide dismutase-2, mitochondrial; TCP1, T-complex locus.

Methods

Materials
Analytical grade chemicals were supplied by BDH Chemicals (Poole, UK). Phenylmethansulfonyl fluoride (PMSF) and iodoacetamide (IAA) were supplied by Sigma Pharmaceuticals (Sydney, Australia). Glutardialdehyde was supplied by Merck Sharp and Dohme (Sydney, Australia) and urea by Gibco BRL Life Technologies (NY, USA). Pharmalyte pH 3–10, Non linear (NL) Immobiline™ DryStrip (IPG) kits loading cups and ExcelGel™ precast 12–14% acrylamide SDS-gradient gels were all supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). Melanie II 2D PAGE software was obtained from Biorad (Hercules, CA, USA). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA).

Tissue samples
Brain tissue was obtained from the NZ Neurological Foundation Human Brain Bank, at the Auckland School of Medicine. The brains were removed post mortem, dissected into anatomical areas and stored at –80°C. The diagnosis of schizophrenia for tissue donors was confirmed retrospectively by a review of clinical notes, according to DSM-IV criteria, by two independent psychiatrists.

Tissue homogenisation
For analytical 2DE, samples of the hippocampus (150–400 mg) were homogenised, in a ground glass homogeniser, pre-cooled on ice in lysis solution (9 M urea, 2% v/v Triton X-100, 2% v/v Pharmalyte pH 3–10, 0.13 M DTT, 8 mM PMSF; 3.5 μl of lysis solution per mg of tissue). To increase the amount of protein, for preparative 2DE, samples were homogenised in a different lysis solution (8 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM Tris, 2% v/v Pharmalyte pH 3–10, 0.13 M DTT, 8 mM PMSF; 3.5 μl of lysis solution per mg of tissue). The homogenate was then centrifuged at $7000 \times g$ for 5 min and the supernatant stored at –80°C.

Two-dimensional gel electrophoresis
2DE was carried out using Non Linear (NL) Immobiline™ DryStrip (IPG), loading cups and ExcelGel™ precast 12–14% acrylamide SDS-gradient gels by the method described previously.[1] Samples for preparative 2DE were made up in sample buffer (8 M urea, 2 M thiourea, 1.2% w/v CHAPS, 2% v/v Pharmalyte pH 3–10, 0.065 M DTT). 2DE gels were calibrated using co-electrophoresed markers for Mr. Preparative 2DE gels were Coomassie R-250 stained.[1]

Comparative proteome analysis
Analytical and preparative 2DE gels were digitised at 125×125 pixels cm^{-1} using a Sharp JX-325 scanner and a green/blue filter. Gel spots were detected, quantified, and matched, using the Melanie II software package, v 2.1. For matching, the image size was reduced by a factor of two and the detection parameters were: smooths 2, Laplacian threshold 3, partials threshold 1, saturation 90, peakedness increase 100 and minimum perimeter 10. The matching of multiple spots to one spot was not allowed. Each spot detected corresponded to a protein of unique pI and Mr. The optical density (OD) of spots was proportional to protein concentration. Normalisation, for protein loading between gels, was carried out, by expressing spots as a percentage of the sum of the optical density for all spots detected on a gel.

Characterisation of spots by in-gel protein digestion
Spots were collected from preparative 2DE gels and digested with trypsin as described previously.[1] After digestion, the resulting peptides were separated by reversed-phase high-pressure liquid chromatography, and their amino acid sequence determined by N-terminal sequencing. Peptide sequences were used to probe non-redundant sequence databases at the NCBI PSI BLAST web site www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast.[25]

The Coomassie-stained protein of interest was cut out of each preparative gel and 9–12 spots were pooled, digested with modified trypsin, extracted and prepared for RP HPLC by the method described previously.[1]

Reversed-phase high performance liquid chromatography (RP HPLC)
The peptide mixture from in-gel digests was separated by RP HPLC at room temperature using a Phenomenex Jupiter C18 column (250×2 mm, 5 μm bead diameter) that was equilibrated for 10 min in a 10:1 solvent A (1% v/v TFA): solvent B (0.8 M acetonitrile in 0.085% v/v TFA) mixture. A linear gradient of solvent B was used, increasing from 5% to 70% over 30 min and ramped to 100% for an additional 5 min, at a flow rate of 300 μl min^{-1}.

N-terminal and internal sequence analysis
Sequence analysis was carried out on selected RP HPLC peaks using an Applied Biosystems Precise Sequencer, model 492.

Probability calculation
To calculate the likelihood that of four genes, chosen at random, three would be located on the same chromosome arm we assumed 44 autosome arms, one sex chromosome and equal numbers of genes on each chromosome arm. The calculation was $1/45 \times 1/45 \times 1/45 \times 44/45 \times 4 \times 45 = 1.9 \times 10^{-3}$.

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