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Mitochondrial Involvement in Schizophrenia and Other Functional Psychoses*

S. A. Whatley[1], D. Curti,[2] and R. M. Marchbanks[1,3]

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Gene expression has been studied in post-mortem frontal cortex samples from patients who had suffered from schizophrenia and depressive illness. mRNA was extracted and characterised by translation and separation of the products by 2D gel electrophoresis. Post-mortem artefacts and the agonal experience did not affect the size distribution or amount of specific translation products. Four expression products were specifically reduced in samples from schizophrenics compared with normals. The expression of six products was altered in affective disorder, one in common with schizophrenia, two the same as in schizophrenia but increased. cDNA libraries were produced from the mRNA samples and 5 clones present at abnormal levels in schizophrenia identified by differential screening, isolated and sequenced. All the sequences encode mitochondrial transcripts; four encode mitochondrial rRNA and one the amino acid sequence of cytochrome oxidase sub-unit II. Increased cytochrome oxidase transcripts were found in a further set of mRNA extracts from schizophrenic patients including two who had not received neuroleptic medication. The effects of neuroleptic administration as exemplified by α-flupenthixol compared with the ineffective β-flupenthixol were studied in experimental animals. It was found that 13 out of 28 clones whose levels were altered were mitochondrial in origin including rRNA, COX I & II and the NADH-Q reductase. Those encoding respiratory enzymes were at abnormally low levels as a result of α-flupenthixol administration. Measurements of the enzymic activity of cytochrome c oxidase in post-mortem frontal cortex of schizophrenics did not indicate any differences in overall activity but there was a decreased sensitivity to azide that was abolished by neuroleptics. Studies on NADH-cytochrome c reductase showed that schizophrenics whether medicated or not had a reduced rotenone sensitive activity that was compensated for by increased rotenone insensitive activity. We conclude that changes in mitochondrial gene expression are involved in schizophrenia and probably other functional psychoses.

KEY WORDS: Schizophrenia; affective disorder; mitochondria; gene expression; cytochrome oxidase; flupenthixol; NADH cytochrome c reductase; psychosis; ribosomal RNA.

INTRODUCTION

Functional psychoses are so called because they are thought to arise as a result of disorderly activity of neurons rather than structural or pathological alterations of the brain. The principle functional psychoses are schizophrenia and affective disorder (manic-depressive illness) which together have a prevalence over the lifetime variously quoted at between 2% and 8%. Schizophrenia is characterised by delusional experiences and thought disorder while affective disorder is characterised by extreme states of elation and or depression that are socially disabling. It is commonly supposed that the illnesses are distinct but there is a considerable overlap of sympto-

[1] Department of Neuroscience, Institute of Psychiatry, Denmark Hill, London SE5 8AF, United Kingdom.
[2] Istituto di Farmacologia, Universita di Pavia, Piazza Botta 11, 27100 Pavia, Italy
[3] Address reprint requests to: R. M. Marchbanks (Tel.: 0171-703-5411 X3263, fax 0171-277-1390).
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matology and some authorities have suggested (1) that they are but the two ends of a common spectrum of mental dysfunction. Among the reasons given by Crow (1) are that there is no distinct bimodality of symptoms nor of outcome and that there is considerable genetic overlap both within different generations and among sibs in the same family.

Studies on the biological basis of the psychoses have turned up many, sometimes contradictory clues but the substantive theories of causation arise from the biological effects of drugs that afford some measure of symptomatic relief. Hence the "dopaminergic" hypothesis of schizophrenia because neuroleptic medications are found to affect various parts of the dopamine neurotransmitter system and the "biogenic amine" hypothesis of affective disorder because of the eponymous effects of antidepressants that frequently also affect aspects of the noradrenergic and serotoninergic transmitter systems. Despite extensive investigation the "neurotransmitter" hypotheses have not been found to be particularly predictive and both suffer from the anomalous disjunction between the 2–48 hr timescale of biological effect and the onset of therapeutic effect after 2–3 weeks (2). The explanation usually offered is that some process of enzyme or receptor induction has intervened between drug administration and therapeutic effect.

The studies we wish to describe started from this background of dissatisfaction with the neurotransmitter hypotheses and were made possible by the general availability of recombinant DNA technology in the 1980's. The well known genetic liability to schizophrenia and affective disorder offered two avenues for exploitation by molecular biology. If there was a nuclear gene it would in principle be detectable by genetic linkage analysis and characterisable by DNA sequencing. The other approach was to analyse the mRNA products of gene expression in schizophrenia and then work backwards to characterise the gene and forward to characterise the protein translation products. The second method seemed to us epistemologically preferable in that experimentation could not be biased by prior assumptions about biochemical causation. Despite this the funding agencies made clear their preference for the genetic linkage approach and for reasons which any working scientist in the UK environment will understand we, along with many others undertook molecular genetic linkage studies on families multiply affected with either affective disorder or schizophrenia and often both. A fairly intensive study of those parts of the human genome that code for "candidate" genes (i.e the dopamine and biogenic receptors and related enzymes) has not so far revealed a genetic linkage that is reproducible under extended scrutiny (3–5). There could be many reasons for this but an obvious one might be that genes such as those in mitochondria that do not segregate and recombine will not be detected by linkage analysis.

The rest of this article will be concerned about the results we obtained by following through the second approach; that is, searching for the genes that are expressed differently in patients suffering from psychosis. The first stage of gene expression is the transcription and editing of coding sequences to mRNA. mRNA may be analysed by translation to protein which can then be separated by 2-D electrophoresis or c-DNA can be obtained by reverse transcriptase and the c-DNA sequences cloned and characterised. Initially we chose characterise the mRNA by translation, mainly in order to satisfy ourselves that the mRNA from post-mortem brain was not so hopelessly degraded as to confound the whole approach.

EXPERIMENTAL PROCEDURE

Total cellular RNA was extracted from frontal cortex obtained post-mortem from 10 normals, 15 schizophrenic patients of which 5 had not received medication and 5 depressed patients. Of the brain samples 24 were from the MRC Brain Bank, Newcastle General Hospital and a further three schizophrenic and three normal samples were provided from the Institute of Psychiatry brain bank. Because of varying tissue availability the same samples were not always used in each study.

Diagnosis of the patients was carried out by means of DSM-III, death was from various causes and the post-mortem delay ranged from 3 to 84 hours. The sample set is described in Table I.

Translation Studies. Poly(A) containing messenger RNA was obtained from total cellular RNA by oligo-(dT)-cellulose chromatography. The messenger RNA was used to direct the in vitro synthesis of proteins containing [^{35}S]methionine by a lysed reticulocyte system. The translation products were analysed by two-dimensional gel electrophoresis and an IBAS computerised image analysis system used to evaluate the integrated grey value of individual expression products (6,7).

Differential Screening and Cloning. Differential screening allows the identification of genes whose relative transcript levels are altered between two tissue types (8). cDNA libraries were made using reverse transcriptase from five of the schizophrenic and five of the normal mRNA samples selected for their superior biological activity in the translation experiments. Double stranded cDNA was cloned (9) into λgt10 after the addition of EcoRI linkers, and infected into E.coli strain C600 hfl using a commercial packaging extract. 20,000 recombinant phage were plated out and duplicate plaque lifts made using Hybond-N nylon membranes. Plaque lifts were differentially screened by hybridising alternately to ^{32}P dCTP cDNA synthesised by reverse transcriptase from schizophrenic and normal frontal cortex mRNA. Following autoradiography, 64 differentially expressed clones were identified by visual comparison of the plaque lifts. The differentially expressed clones were lifted, amplified by PCR digested and separated by electrophoresis on agarose and transferred to a Hybond-N membrane. The DNA on the membrane was then sequentially hybridised to cDNA synthesized from four individual schizophrenic and four individual normal mRNA samples using the same protocol as the pri-

Table I. Details of Post-Mortem Frontal Cortex Samples from Normals and Schizophrenics

Group	Sex	Age of onset	Age of death	PM delay (h)	Cause of death	Medication	
						Psychotropic	Other
Normal							
	M	—	64	8	IHD	N	—
	F	—	59	28	CA	N	—
	M	—	85	22	IHD	N	—
	F	—	90	60	IHD	N	OP
	F	—	81	25	CA	N	OP
	F	—	78	18	CA	N	—
	M	—	63	31	IHD	N	—
	M	—	51	15	COAD	N	—
	M	—	51	14	S	N	—
	M	—	63	27	IHD	N	—
	M	—	38	34	IHD	N	—
Schizophrenic							
	M	32	66	68	IHD	Y	—
	M	20	60	41	IHD	Y	—
	F	25	71	48	BP	Y	BZP,Li
	M	39	84	29	BP	Y	—
	M	35	47	36	IHD	Y	BZP
	F	31	71	64	CA	Y	—
	M	30	72	50	BP,CA	Y	OP
	M	28	78	60	IHD	Y	—
	M	?	70	48	BP	Y	—
	F	24	76	97	BP	Y	—
Unmedicated Schizophrenic							
	F	30	77	78	BP	N	—
	M	23	66	9	BP	N	—
	M	39	71	28	SEPT	N	—
	M	30	63	84	GITB	N	—
	F	30	87	7	BP	N	—

KEY: IHD, Ischaemic heart disease. CA, carcinomatosis. COAD, chronic obstructive airways disease. S, sudden death. BP, bronchopneumonia. SEPT, septicaemia. OP, opiates. BZP, benzodiazepines. Li, Lithium. GITB, gastrointestinal tract bleeding.

mary screen. Following each hybridisation the filter was 'stripped' before proceeding to the next. Only one master filter was used during the secondary screening process, to eliminate such variable factors as PCR yields and DNA transfer. The results for each individual clone were therefore comparable despite differences in specific activity of the probe, exposure time and slight variations in the stringency of washing. Autoradiograms were examined using an image analysis system and the integrated grey value (IGV) was determined within a constant area delineating each spot. IGVs were normalised by expressing each as a fraction of the average of all the signal intensities on the autoradiogram. cDNA inserts were subcloned into either Bluescript (Stratagene) or pCR II (Invitrogen) and double stranded sequencing was performed using SP6 and T7 primers and the Pharmacia T7 polymerase sequencing kit.

Biochemical Assays. The activity of cytochrome oxidase (COX) can be measured by the reduction of cytochrome c, uptake of oxygen or by the oxidative polymerisation of diaminobenzidine. Since the changes observed in COX mRNA might be associated with one or other of the different manifestations of enzymic activity all three measures have been carried out. Polarographically COX activity was measured using a Gilson oxygraph with a Clark-type oxygen electrode at 37°C. In the assay chamber the homogenate (250 µl 1:20 w/v) was added to the medium (Na_2HPO_4/KH_2PO_4 50 mM, pH 7.0, ascorbate 1.5 mM, cytochrome c 25 mM, TMPD 0.6 mM), when necessary, NaN_3 was added in order to inhibit COX mediated oxygen consumption. Cox activity is expressed as $nmol \cdot min^{-1} \cdot mg \cdot prot^{-1}$.

Spectrophotometrically COX activity was assayed by the method of Wharton & Tzagaloff (11) with few modifications: the assay mixture consisted of Tris/HCl 0.2 M pH 7.0 and 0.01% lauryl-D-maltoside (a non-ionic detergent). An aliquot (40 µl) of homogenate (1:20 w/v) were added to the mixture and the reaction was started with 0.07 ml of 1% reduced cytochrome c solution. The enzyme was measured with a Cary 3 spectrophotometer at 37°C.

COX assay by polymerisation of diaminobenzidine (DAB) is based on the histochemical method of Seligman et al. (12) in which cytochrome oxidase (COX) transfers electrons from cytochrome C via the COX-H_2O_2 intermediate to 3,3'-diaminobenzidine (DAB) to give a black insoluble product. In this procedure the product is filtered off and can be estimated quantitatively with an electronic scanning device. Catalase is not required nor is it active in the assay. Cytochrome oxidase from Sigma (1 µl approximately equal to .25 units) or 2–10 µl of a 20% (w/v) brain homogenate in 50 mM potassium phosphate was added to 100 µl of a buffer composed of 1 mM EDTA in K-phosphate 20 mM pH 7.4 with 0.1% lauryl-D-maltoside. To this was added reduced cytochrome c (from horse heart, Sigma C-7752) to a final concentration of 100 µg/ml and the reaction started by the addition of a freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (Sigma) to a final concentration of 0.5 mg/ml. Incubation was at room

temperature for 10–100 minutes depending on activity after which 20 ml of the reaction medium was filtered under suction in the wells of a Biorad filter manifold loaded with Whatman GF/F glass fibre filter paper. After the black precipitate had filtered off it was washed with 2 × 200 µl phosphate buffered saline and the filter paper dried. Quantitation was carried out with a Logitech scanner and the darkness of each spot converted to numerical estimate (integrated grey value, IGV) using Quantiscan 2.1 software. Originally the filters were calibrated with Indian ink but the stability of the measuring equipment appears to render this unnecessary (coefficient of variation = .04) and a standardisation factor of 3.5 IGV per nmole unit of purified bovine cytochrome oxidase from Sigma was adopted. There is some uncertainty about the exact stoichiometry of the polymerisation reaction but it is clear that the yield is substantially below the theoretical expected if all the peroxyl intermediate was diverted to react with DAB. COX as measured by this method was linear with time in the range 10–200 min, linear with amounts of pure enzyme in the range 0.01–0.5 units; activity of the pure enzyme was 95% dependent on the addition of reduced cytochrome C, it was inhibited 98% by 1 mM cyanide and the coefficient of variation of repeated measurements was .09. Protein in the 20–200 µg range in these samples could be readily measured by a 20 min incubation with 100 µl 0.2% amido-Schwarz in 10% acetic acid followed by filtration and comparison with a set of standards similarly treated.

NADH-Cytochrome Reductase was measured as the change in optical density of cytochrome c at 550 mµ and is expressed as nmole.min^{-1}.mg prot^{-1}. The cytochrome c concentration was 40 µM and 100 nmoles of NADH was added to initiate the reaction.

Citrate Synthase (CS) was assayed by the method of Sugden and Newsholme (13) The assay mixture consisted of Tris/HCl buffer 50 mM pH 7.5, Triton X-100 0.5%, oxaloacetate 0.5 mM, 5-5'-dithio-bis-(2-nitrobenzoic) acid (DTNB) 0.2 mM. An aliquot (20 µl) of homogenate (1:20 w/v) were added to the mixture and the reaction was started with acetyl-CoA 0.1 mM.

Protein content was assayed by the method of Lowry et al. as modified by Schacterle and Pollack (33).

RESULTS

Translation Experiments. In preliminary experiments (6) it was found that the yields of total and mRNA did not vary within the illness groups and that the amount of RNA extracted did not vary according to the post-mortem delay time. More importantly the biological activity of the mRNA as shown by its ability to direct protein synthesis was not significantly different in the illness groups, nor did it decrease significantly with post-mortem delay time. An informal inspection of the electropherograms did not reveal any systematic changes in the size distribution of products between illness groups or post-mortem delay time. We concluded from this that it should be possible to analyse differences in individual gene expression by this technique without undue interference from post-mortem breakdown or gross illness related changes in the mRNA. Changes in specific gene expression products were therefore analysed in more detail (7). The intensity of 74 well resolved proteins on the autoradiograph was measured with an interactive image analysis system. The intensity was measured in autoradiographs from each of 10 schizophrenics, 5 depressed patients and 10 normals. The average intensity of seven expression products was found to be significantly changed ($p < 0.05$) in the illness groups (Table II).

The expression of four proteins was found to be decreased in the schizophrenic group, one of these proteins (#32) was also decreased in the patients with affective disorder. Two of the other proteins (#66 & #67) were decreased in schizophrenia and also were found to be increased in affective disorder. Changes were also found in three other expression products (#7, #10 & #53) in the frontal cortex of depressed patients. Because of the spread of agonal and post-mortem delay times it was possible to establish that none of these changes showed significant correlation with either. The effects of medication are difficult to analyse because 9 out of the 10 schizophrenics had received phenothiazines, but with the exception of one expression product the single drug free patient was found to show the same pattern of expression as the medicated schizophrenics.

The expectation by chance alone of finding expression products that are changed in both schizophrenia and affective disorder is rather low: p(3 sz out of 6 ad) < .0026 and p(3 ad out of 4 sz) < .0019. We believe that the existence of commonly changed expression products in both illness groups gives support to the clinically derived proposal that schizophrenia and affective disorder are different aspects of the same dysfunction. The translation studies have been helpful in providing quantitative information that there are gene expression changes and that they are not likely to be artefactual but it is not easy to identify the qualitative nature of the change because there is insufficient product for protein sequence analysis. Also because the autoradiographs show the pattern of proteins before post-translational modification they are not readily comparable to the patterns obtained by 2-D electrophoresis of brain proteins.

Differential Screening and Cloning. mRNA populations in post-mortem brain of schizophrenics were compared to normals by differential screening, a protocol that allows the identification of genes whose relative transcript levels are altered between two tissue types (8). 3 clones (B2, A36 and A35) were significantly under represented ($p < 0.05$) in the cDNA from the four schizophrenic brain samples (9). One clone (B24) was consistantly increased in schizophrenic compared to normal frontal cortex though the level of differential expression was not statistically significant. Clone (B25) demonstrated an increase in abundance in schizophrenic frontal

Table II. Changed Translation Products in Schizophrenia and Affective Disorder

Protein species	pI	M (kDa)	Normal	Schizophrenic	Depressive
7	6.2	38	0.122 ± 0.073	0.142 ± 0.061	0.671 ± 0.129[b]
10	6.2	34	0.234 ± 0.053	0.212 ± 0.077	0.014 ± 0.002[c]
32	5.8	33	0.420 ± 0.089	0.029 ± 0.013[d]	0.129 ± 0.036[a]
42	5.8	26	0.390 ± 0.102	0.072 ± 0.034[b]	0.371 ± 0.066
53	5.7	17	0.506 ± 0.143	0.522 ± 0.092	1.620 ± 0.182[c]
66	7.1	35	0.600 ± 0.143	0.006 ± 0.001[d]	1.401 ± 0.233[a]
67	6.1	23	0.280 ± 0.082	0.007 ± 0.001[c]	0.942 ± 0.120[c]

The integrated grey values were determined by computerised image analysis and normalised with respect to the average intensity on each 2 dimensional autoradiograph. Values given are the means and SEM of 10 normals, 10 samples from schizophrenic patients and 5 samples from patients with unipolar depression. Only those species are shown that changed significantly ($p < 0.05$) in the illness groups. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.005$ & [d]$p < 0.001$.

Table III. Identification of Differentially Expressed Genes in Schizophrenia

	Relative Amounts of cDNA			
Clone	Normal	Schizophrenic	Drug free	Identity on sequencing
B24	2.76 ± 0.16	3.01 ± 0.24	4.5	MtDNA 692-1010 12s RNA
A36	1.97 ± 0.13	1.38 ± 0.27[a]	1.4	MtDNA 2002-2160 16s RNA
B2	1.14 ± 0.07	0.74 ± 0.10[b]	.4	MtDNA 2127-2400 16s RNA
A35	0.52 ± 0.04	0.34 ± 0.07[a]	.2	MtDNA 2829-3232 16s RNA
B25	3.79 ± 0.36	4.26 ± 0.47	5.7	MtDNA 7779-8294 COX II
	Relative amounts of mRNA			
Probe				
COX II 7779-8294	1.27 ± 0.19	2.26 ± 0.33[b]	2.66 ± 0.26[c]	
	Relative amounts of MtDNA			
total MtDNA				
5651-14490	.913 ± 0.16	1.07 ± 0.05	1.34 ± 0.045	

In the top panel is given the normalized integrated grey value and identity by sequence of the 5 out of 64 original clones selected for differential screening. Figures given are the mean and SEM (N = 4) for each group but the "drug free" schizophrenic sample is presented separately.
In the second panel is given the results of a northern blot analysis for COX II mRNA of a further set of samples from 5 normals, six medicated and two drug free schizophrenics all done in triplicate. In the third panel is given the results of probing a southern blot of unrestricted DNA extracted from 5 normals, six medicated and 2 drug free schizophrenics with mitochondrial sequences. An oligo-dT probe was used to check that standard amounts of cDNA and mRNA were applied to the blots. [a]$p < 0.05$, [b]$p < .01$, [c]$p < .005$.

cortex and was even further increased in the only drug free schizophrenic sample in the study.

The clones were identified on the basis of nucleic acid identity to known sequences in the EMBL and Genbank databases. Table III gives details of the quantitation and identification of the clones. All five clones were found to be derived from mitochondrial (mt) DNA, those demonstrating reduced expression in the schizophrenic brain (B2, A36 and A35) all derived from the 16s rRNA whilst the marginally increased cDNA (B24) derived from the immediately preceding 12s rRNA. B25, whose increase in the drug free sample was noted encodes a partial sequence of the mitochondrial enzyme cytochrome oxidase subunit II. In order to verify the results from the screening experiment cytochrome oxidase II mRNA levels were quantitated in a further sample set of frontal cortex tissue using a dot-blot assay. Results are given in the second panel of Table III and show that expression of cytochrome oxidase II is significantly increased in the schizophrenic brain with an even greater increase in the two drug free samples then available to us. Also shown in Table III panel 3 is a comparison of mtDNA between frontal cortex tissue from schizophrenics and normals. Except for the two drug free samples the amount of mtDNA is not significantly different indicating that gross differences in the number of mitochondria or copies of mtDNA would not explain the cloning result. To check that the original library had not been artifactually enriched in mitochondrially derived transcripts it was re-screened with human placental mitochondrial DNA. 42 out of 4758 clones were positive. When the library was screened with a 1kb fragment of

Table IV. Clones Differentially Expressed by α-Flupenthixol

Identity of gene fragments	Number down regulated	Number up regulated
Mitochondrial genes :		
12s RNA		1
16s RNA	2	
NADH-Q reductase (ND2)	3	
Cytochrome oxidase (COX I)	2	
Cytochrome oxidase (COX II)	5	
ATPase 6	1	
Nuclear genes :		
NADH-b_5 reductase	1	1
slgA glycoprotein	1	
defensin		1
connexin		1
Unknown:	8	2

The probability by chance of selecting 14 mitochondrial clones out of 29 given a library frequency of 1% is about 6.6×10^{-21}.

actin cDNA 38 out of 2000 plaque forming units were recognised.

Gene Expression Induced by Neuroleptic Medication. In the light of these results it seemed important to establish independently the effects on gene expression of neuroleptic medication. cis(α)-flupenthixol was selected for this study because its geometrical isomer trans(β)-flupenthixol is inactive therapeutically and is less than a thousand times as active as a dopamine receptor antagonist so that it can serve as a useful experimental control. Doses in the therapeutic range of 5 mg/kg daily were given to rats, for a period of three weeks, after which forebrain mRNA was extracted and checked by 2D translational analysis (10). cDNA libraries were constructed and 64 differentially (as between α- and β-flupenthixol) expressed clones were isolated. Of these 29 have been sequenced by Simon Jones (personal communication) with results shown in Table IV.

The most striking feature of the results is that 14 out of the 29 clones sequenced are of mitochondrial origin. With a library frequency of mitochondrial sequences of about 1% the probability of this being a chance observation is rather less than 10^{-20}. Predominant among the mitochondrial sequences are those coding for cytochrome oxidase subunits I and II and the NADH-Q reductase, all of which are down regulated by α–flupenthixol. The mitochondrial genome is circular with transcription proceeding from a well defined origin to give 12 separate transcripts roughly corresponding to known mitochondrial rRNA or polypeptides. It is interesting that all the differentially expressed mitochondrial transcripts are among the first six to be transcribed. The nuclear gene coding for NADH-b_5 reductase was identified twice but with no consistent direction of regulation.

Biochemical Studies on Mitochondrial Enzymes. Although the presence of a differentially expressed mitochondrial transcript does not necessarily indicate the existence of changed levels of the functional enzyme it seemed of interest to investigate initially the concentrations of mitochondrial enzymes involved in Complex IV (Cytochrome oxidase) and Complex I (NADH-Q reductase) since their coding sequences were represented among those whose expression was altered both in schizophrenia and as a result of neuroleptic administration.

Cytochrome Oxidase (Complex IV). This enzyme was measured in post-mortem brain samples by three different methods. The results are shown in Table V. No significant differences were found by any of the methods in the amounts in medicated or drug free schizophrenics. Although there was not found to be any illness related change in the activity of the enzymes there was found to be a change in the sensitivity to azide.

As might be expected azide inhibited the activity of the enzyme in all cases but there was found to be a small but consistent decrease in its inhibitory effectiveness in tissue from unmedicated schizophrenics. The differences were found by all three methods of COX measurement. These differences could be due to residual non-specific peroxidative or oxygen uptake activity but it would seem unlikely that both effects would co-exist. The inhibitory effect of azide was complicated by a pronounced non linearity in that higher concentrations of azide were less effective than would be predicted by the usual models of enzyme inhibition. The non linearity was found by all three methods of COX measurement so a non trivial explanation was sought. Hill plots ($\log(v_o/v_i - 1)$ vs $\log [I]$) gave negative co-operativity coefficients of 0.3–0.5 which could be explained by the interaction between the 2 haem groups in cytochrome oxidase capable of reacting with azide. The inhibitory constant with respect to azide is higher in drug free schizophrenics compared with normals or medicated schizophrenics indicating a decrease in the effectiveness of the inhibitor against some proportion of the cytochrome oxidase activity in samples from those patients who had not been exposed to neuroleptics.

NADH-Cytochrome c Reductase (Complex I–III). The other mitochondrial enzyme which is of immediate interest because of its sensitivity to induction by flupenthixol is the NADH-Q reductase (Complex I). This was

Table V. Cytochrome Oxidase Activity and Its Inhibition by Azide in Frontal Cortex from Normal and Schizophrenic Subjects

	Normals	Schizophrenic	Drug free
COX polarographic (O_2uptake)	46.75 ± 2.77 (10)	45.03 ± 2.03 (9)	47.36 ± 5.24 (4)
COX spectrometric (cyt c oxidation)	23.6 ± 2.8 (10)	21.6 ± 1.9 (9)	29.6 ± 5.6 (4)
COX DAB method (polymerised DAB)	16.5 ± 2.7 (10)	12.9 ± 1.4 (10)	15.8 ± 5.8 (5)
K_i azide (mM)	0.747 ± 0.066[a]	.662 ± 0.033[b]	1.09 ± 0.147

The first three rows of the table show COX activities measured as described in the Experimental Procedure section with means and SEM & N relating to the number of patients in each group from whom samples were analysed. In the bottom row the inhibitory constants were determined using azide concentrations in the range 0.1 mM to 20 mM and the expression $K_i = [azide]^{0.4}/(v_i/v_0 - 1)$. Because of tissue shortages not every sample was tested at each inhibitor concentration by each method. The figures given are the means and SEM of 50 measurements on normal and schizophrenic samples and 24 unmedicated schizophrenic samples. [a]$p < .05$, [b]$p < .01$ drug free schizophrenics with respect to normals and medicated schizophrenics respectively.

Table VI. NADH-Cytochrome C Reductase Activity in Frontal Cortex of Normals and Schizophrenic Subjects

	Normals	Schizophrenic	Drug free
Total activity	6.53 ± .57	7.38 ± .78	6.02 ± .81
+ Rotenone (10 μM)	−0.26 ± .98	3.72 ± 1.1**	2.47 ± 1.2
Respiratory chain reductase activity	6.82 ± .95	3.66 ± .82**	3.53 ± 1.2
		Citrate Synthase	
	154 ± 14	161 ± 11	171 ± 17

The figures given are the mean and SEM of duplicate measurements of samples from each of 10 normal, 10 schizophrenic and 5 unmedicated schizophrenic patients. **$p < .01$ for schizophrenics vs normal. The respiratory chain cytochrome c reductase activity was obtained by from the difference between total and rotenone inhibited calculated for each sample. The small negativity of the value for the activity of Normals in the presence of rotenone is not significantly different from zero.

measured as the rate of rotenone sensitive cytochrome c reduction by NADH in the presence of cyanide to prevent reoxidation by cytochrome oxidase. Rotenone sensitivity is usually taken as an indicator of respiratory chain activity and in the Normals all the cytochrome c reducing activity is rotenone sensitive.

However it was found that in schizophrenic patients the rotenone sensitive reducing activity was replaced by a rotenone insensitive route to cytochrome c reduction. This could mean that *either* that the respiratory chain reductase activity is decreased in schizophrenics with a compensating increase in the outer membrane (b_5) reductase *or* there may be change in the properties of Complex I such that it is not sensitive to rotenone. The same pattern is seen in unmedicated schizophrenics so that the rotenone insensitivity is unlikely to be a drug effect. Also shown in Table VI are measurements of citrate synthase used here as a marker of mitochondrial functional mass. There were no significant differences between the illness groups. Taken in conjunction with the measurements of mitochondrial DNA (Table II) this suggests that there are not differences in the number of functioning mitochondria in psychosis.

DISCUSSION

The results from the cloning experiments indicate a mitochondrial involvement in schizophrenia and since common translational products were found probably also in other functional psychoses such as affective disorder. The cloning procedure is completely free of bias introduced by preconceived ideas of the causation of the illness. It is also catholic in that the complete range of biochemical function coded for in the genome is accessible to inspection. Since there is no reason to suppose that mRNA coding for different sequences degrade at different rates changes in labile metabolites that would normally be lost can be detected by inference. The results we have obtained could not reasonably have been expected by chance ($p < 10^{-10}$) but were unexpected in that they did not support conventional views about the causation of schizophrenia. The response of the editors of journals and referees of grant applications has been a salutory experience. With a few honourable exceptions they have evaluated the results by appeals to received wisdom rather than reasoned scientific argument.

The question of whether the cloning procedure has produced artefactual results is central to the evaluation of our results. Mitochondrial DNA exists in about 10 copies per mitochondria of which there are about 100–200 per cell. Thus mitochondrial DNA is about 1% of total cellular DNA and if its rate of transcription is overall about the same as nuclear DNA one would expect about 1% of the cDNA in the library to be mitochondrial in origin. This is what we found in our own cDNA libraries. If these 1% DNA sequences all have an equal chance of being selected then the probability of selecting 5 by chance is 10^{-10}. But could the relatively high frequency of mtDNA sequences have biased the selection during the differential hybridisation procedure? We are unable to suggest a plausible mechanism that would select both up and down regulated clones from the library. However there is an empirical test; actin sequences were present in our library with a frequency of about 2% yet none were selected by the differential screening procedure.

The results presented here are open to the usual arguments raised against all post-mortem analysis; such as the possibility of artifacts due to post-mortem delay, and agonal state. All the mRNA samples used in the study had been tested for integrity and although post-mortem delay times were greater for schizophrenic patients compared to normal it had been shown (7) that post-mortem delay times do not significantly affect mRNA integrity or activity. In addition it is unlikely that differences in post mortem delay times would introduce such a consistent selection of mitochondrial sequences. It is possible that we have detected an agonal state effect, however medical reports obtained indicate that hypoxia was short term for all individuals examined. Mitochondrial deletions are known to occur in inceasing numbers in the brain with age (14), however the samples used in this study were age-matched and it is therefore unlikely that we have detected an age-related phenomenon. We therefore consider that the results presented reflect real changes associated with the RNA populations of the brain samples examined rather than chance observation.

That the results might be due to the medication or lifestyle of the patients is more difficult to evaluate but we now have some evidence on the effects of drugs. α-Flupenthixol caused a decrease in the expression of most of the mitochondrial transcript. The exception was the 12sRNA which was increased in our schizophrenic sample, and by α-flupenthixol in rats. Expression of the COX II gene was increased in schizophrenics, more so in drug free and found to be down regulated by α-flupenthixol. A reasonable explanation would be that overexpression of COX II is the usual accompaniment to schizophrenia and is reversed by neuroleptic medication. However we would not want to suggest that this is necessarily the *modus operandi* of therapeutic effect. We have no information on the occurrence of smoking in our sample but there is known to be a high incidence among schizophrenics generally where it has a phenothiazine supplanting effect (15). This would tend to obscure the differences we have observed rather than cause them. Finally, there are gender differences in age of onset and clinical severity of schizophrenia. Our sample was disproportionally male so that the possibility must be conceded that we have observed only a gender biased selection of changes. But since our samples originated from elderly patients where gender differences are minimal we do not see this bias as disabling.

It is hardly surprising that no changes in the enzymic activity of cytochrome c oxidase were found in frontal cortex from schizophrenics. The enzyme is so central to oxidative metabolism that any gross alteration would have been manifested as a substantial metabolic and pathological derangement as is seen in some of the mitochondrial encephalomyopathies in which functional mutations of COX are known to occur, for example, Kearns-Sayre syndrome and more recently (16) for the Leber's disease. In fact a small though significant decrease has been seen in the sensitivity of COX activity to azide in unmedicated schizophrenics. This could of course be due to non-specific oxygen uptake, peroxidative activity or cytochrome c reducing substances or non-specific effects on COX for example lipid occlusion. But it is also possible that changes in the mitochondria of schizophrenics restrict access of azide to the bimetallic centre Fe_{a3}–Cu_B of COX at which it is known to inhibit (17).

Since our experiments on COX were completed we have read the results of Cavalier's studies on the enzyme in the post-mortem brains of patients with Alzheimer's disease and with schizophrenia (18). These authors assert that cytochrome oxidase activity is decreased in the brains of schizophrenics. As regards the frontal cortex this claim is not borne out by the data they present. An examination of the age distribution of their normal and schizophrenic group as presented in *their Table I* shows that their schizophrenic group was significantly older at death than their normal group. (sz 82.5 ± 4.1 N = 10, normal 70 ± 5.2 N = 7). The corresponding activities of COX are 7.1 ± .47 and 12.5 ± 2.9 (SEM). Neither of the differences reaches a significance level greater than $p < .1$ (contrast *their fig 2*) but if a simple linear regression is carried out on the combined frontal cortex data for normal and schizophrenics given in their table a significant negative correlation of COX with age is

found. (COX vs age: $b = -.205 \pm .085$; $p < .05$). Our own data does not show this because the age at death of the normals and schizophrenics is well matched. (Table I).

The most marked change in the enzymic composition of mitochondria from schizophrenics has been found in the Complex I region. It is not clear yet whether we have observed a loss of sensitivity to rotenone or a deficit in Complex I with a compensatory increase in outer membrane reductase also known as cytochrome b_5 reductase. It is intriguing but inconclusive that the expression of the cytochrome b_5 gene appears to be flupenthixol sensitive. If Complex I throughput is attenuated in schizophrenia then the increased b_5 reductase may be an adaptive response to oxidise excess NADH so that it can be and recycled.

Mitochondrial dysfunction is not usually associated with schizophrenia or affective disorder, but has now been reported in several mental disorders. Decreases of 50–60% in COX I & III mRNA have been observed in Alzheimer's disease (19) and a 20–30% decrease of COX activity has been found in frontal regions of post-mortem brain from patients suffering from Alzheimer's disease (20,21) where a decreased metabolic rate of glucose has also been revealed (22) by positron emission tomography (PET scan). Changes in Complex I have been shown by (23) in Parkinson's disease.

Nerve cell activity is highly dependent on energy metabolism and many neurotransmitter systems are either directly or indirectly affected by mitochondrial metabolism. Cytochrome oxidase and the NADH-Q reductase (Complex I and IV) are key enzymes in the respiratory chain producing metabolic energy. These enzymes are tightly controlled by nuclear factors so that an increase in their mRNA could be the result of a regulatory response to functional deficiencies. A mitochondrial involvement in schizophrenia was first proposed by Takahashi (24) who showed that aerobic glycolysis was lowered in post mortem brain from schizophrenics. Several studies by positron emission tomography (PET scan) have revealed a decreased oxidative metabolism in the schizophrenic frontal cortex (25,26). At present there is debate as to whether the decreased oxidative metabolism in schizophrenia represents a causal factor or simply a secondary effect of the disorder. The molecular evidence presented here supports the theory that cerebral oxidative metabolism is altered in schizophrenia brain, is a primary cause and has a genetic basis. Auto-antibodies to a product of one of the nuclear genes controlling mitochondrial replication (26) are correlated with the psychotic sequelae of systemic lupus erythematosus (28,29).

Mitochondrial DNA is maternally transmitted and even though there is evidence of increased female compared to male transmission of schizophrenia (30,31) completely maternal transmission of schizophrenia is not generally observed. This could be because mitochondrial transcription and function is under partial nuclear control and a complex pattern of inheritance would result if mitochondrial and nuclear factors interact (32). Such a pattern would not be inconsistent with the complex inheritance pattern observed in schizophrenia.

Turning to broader issues; the evidence we have obtained indicates that in schizophrenia there are changed concentration of mitochondrial transcripts and that neuroleptic drugs also cause changes in the concentration of the same mitochondrial transcripts usually in the opposite direction. The action of neuroleptic drugs is thought to be mediated by the dopamine receptor which is itself linked to the cyclic AMP second messenger system. There are numerous examples of cyclic AMP modulation of gene transcription and it is well known that it also modulates metabolic activity, particularly glycolysis at the 6-phosphofructose-1-kinase stage. Clearly this chain of events needs to be substantiated by direct experimentation but it is not at this stage an unrealistic suggestion.

The changed concentration of transcripts in unmedicated schizophrenics could be due (i) to an abnormality in a nuclear gene that controls mitochondrial replication or transcription of the mitochondrial genome or (ii) an abnormality in a nuclear gene coding for one or other of the accessory mitochondrial proteins which as a secondary effect causes changes in the mitochondrial transcripts. (iii) an abnormality in the mitochondrial genes controlling the replication and transcription apparatus itself. (iv) an abnormality in the mitochondrial genes coding for the respiratory chain. None of these can be strictly ruled out but on the limited information we have at the moment we think it is less likely that genes either nuclear or mitochondrial that control replication or transcription are involved because measures of total mitochondrial mass, for example, citrate synthase or total mtDNA do not appear to be changed by schizophrenia. This leaves the possibility of abnormalities in mitochondrial proteins themselves either nuclear or mitochondrially coded. Screening for single DNA mutations can be carried out on the scale of the mitochondrial genome and this is currently under way. However many of the nuclear coded mitochondrial proteins have not yet been identified at the DNA level so that further biochemical characterisation of the mitochondrial changes in schizophrenia is also a necessary approach that is currently underway.

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