J Neural Transm (2007) 114: 665–674
DOI 10.1007/s00702-006-0581-8
Printed in the Netherlands

Journal of Neural Transmission

Quantification of total mitochondrial DNA and mitochondrial common deletion in the frontal cortex of patients with schizophrenia and bipolar disorder

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Received: June 30, 2006 / Accepted: September 25, 2006 / Published online: January 3, 2007

Summary Data published during the last decade are suggestive of a role for mitochondrial dysfunction in the pathogenesis of schizophrenia, bipolar disorder and other psychiatric diseases. In order to determine if the mitochondrial deficits reported in the literature are caused by abnormalities in the mitochondrial DNA of psychiatric patients, we quantified mitochondrial DNA (mtDNA) levels and the 5 kb common mitochondrial deletion (CD) in postmortem frontal cortex tissue. The mitochondrial CD and mtDNA levels were measured in tissue obtained from the frontal cortex (Brodmann Area 46) of 144 individuals (45 patients with schizophrenia, 40 patients with bipolar disorder, 44 controls, and 15 patients with major depression). These variables were measured using newly developed SYBR green and TaqMan real time PCR assays.

Both the TaqMan and the SYBR green assays gave similar results. There was no statistically significant difference for the quantity of the common mitochondrial deletion between controls and patients. We also did not detect a difference in the mtDNA levels amongst the diagnosis groups. There were statistically significant differences for the evaluated parameters for smokers, schizophrenic patients on antipsychotic drugs at time of death, and bipolar patients with antidepressant use and alcohol abuse. Based on this study and other reports, we conclude that neither the common mitochondrial deletion nor changes in mitochondrial DNA levels are likely to account for the mitochondrial changes associated with bipolar disorder or schizophrenia. The effect of premortem agonal factors and medication on mitochondrial dysfunction still needs further elucidation.

Keywords: Mitochondria, common deletion, schizophrenia, bipolar disorder, major depression, DNA, real-time-PCR

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Introduction

Mitochondrial dysfunction is recognized as the underlying cause for a growing number of sporadic and inherited human diseases called ''mitochondrial disorders'' (for reviews see Wallace, 1994, 1999; Zeviani and Di Donato, 2004; Beal, 2005; Taylor and Turnbull, 2005). Mitochondrial disorders typically occur in postmitotic tissues such as skeletal muscle, cardiac muscle, and the nervous system and include a wide range of syndromes, typically affecting various organs simultaneously. These disorders include Chronic Progressive External Ophthalmoplegia (CPEO), Myoclonic Epilepsy with Ragged Red Fibers (MERRF), Mitochondrial Encephalomyopathy with Lactacidosis and Stroke Like Episodes (MELAS), Morbus Leigh, Leber's Hereditary Optic Neuropathy (LHON) and many others. Mitochondrial diseases are caused by a series of distinct pathogenic mutations occurring in the mitochondrial DNA (mtDNA), leading to functional defects in oxidative phosphorylation (OXPHOS) and to the enhanced generation of superoxide radicals. The resulting decrease in cellular energy load (ATP) accompanied by the toxic effects of superoxide radicals and their byproducts (OH, H_2O_2, peroxynitrite) are thought to cause severe dysfunction and eventually lead to cell death. In ''classic'' mitochondrial disorders a single mtDNA mutation accumulates in postmitotic tissue over time before it reaches a threshold level and gives rise to disease. The disease-causing mutations

can be either maternally inherited or sporadic, i.e. somatic mutations. Determining the threshold mutation level for disease is difficult, since a great deal of variability exists amongst tissue and cell types in the number of mtDNA molecules and the proportion of mutant to wild type mtDNA molecules. Functional defects in the end-stage of mitochondrial energy production, especially in the respiratory chain complex 1, are detected in various neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson and Alzheimer disease. However, the mitochondrial defects in these diseases are not caused by a single pathogenic mtDNA mutation. These dysfunctions may be associated with the culmination of a number of different somatic mutations, which may be randomly distributed within the mitochondrial genome and accumulate during aging, due to the action of reactive oxygen species.

Recently clonal large scale deletions have been proven to reach physiologically significant levels in substantia nigra neurons of Parkinson patients (Bender et al., 2006; Kraytsberg et al., 2006). These reports also demonstrated for the first time, that somatic mutations, which are present in trace amounts in brain homogenates, can be found in dissected neuron populations at much higher levels, compatible with severe damage of oxidative phosphorylation. They can thus no longer be regarded as physiologically irrelevant byproducts of oxidative stress.

Mitochondrial abnormalities are also implicated to play a role in the pathology of serious psychiatric illnesses. Several recent microarray studies have reported differential expression of mitochondrial gene transcripts in postmortem brain tissue samples of patients with schizophrenia and bipolar disorder (Konradi et al., 2004; Prabakaran et al., 2004; Altar et al., 2005; Iwamoto et al., 2005). Iwamoto et al. (2005) reported a global down-regulation of mitochondrial gene expression in the frontal cortex of both schizophrenic and bipolar patients. The authors attributed their findings to the effects of medication and did not believe that mitochondrial dysfunction is a causative factor for either schizophrenia or bipolar disorder. Prabakaran et al. (2004) used a multifaceted approach to examine changes in the postmortem frontal cortex at the transcript, protein and the metabolic level. Their combined analysis implicated several mitochondrial genes to be involved in these psychiatric disorders. The authors also claimed that the mitochondrial changes they observed were independent of drug effects and concluded that mitochondrial defects may contribute to the pathophysiology of psychiatric ailments. Konradi et al. (2004) performed microarray analysis in the hippocampus and, like the previous groups, reported a decrease in mitochondrial RNA expression in schizophrenia and bipolar disorder. They hypothesized that the loss of mitochondria-rich cells in the brain such as GABA-ergic interneurons may account for the observed decrease in mitochondrial gene expression. Altar et al. (2005) performed microarray analysis on laser-captured dentate neurons from the hippocampus and found a similar reduction in the expression of mitochondrial genes. These findings were further supported by the significant decrease in COX activity in the caudate nucleus (63%) and the frontal cortex (43%) of schizophrenia patients (Cavelier et al., 1995). Finally, magnetic resonance studies have found a decrease in metabolism in the frontal cortex of schizophrenic patients (Volz et al., 1997) although the results of magnetic resonance studies have been contradictory (Fukuzako, 2001; Keshavan et al., 2000). Conversely, overexpression of COII, one of the three mitochondrial DNA (mtDNA) encoded COX subunits, in the frontal cortex of patients with schizophrenia refutes the case for a global decrease in mitochondrial gene expression (Whatley et al., 1996). Using animals, these authors also demonstrated that the observed effect was unlikely to be caused by antipsychotic drugs, since these drugs tended to induce the opposite effect. Similarly, COII mRNA levels were differentially altered (either up- or down-regulated) in different parts of the basal ganglia, i.e. caudate nucleus, putamen, and globus pallidus in postmortem samples from schizophrenic patients (Prince et al., 1999).

Several groups have reported that mutations in the mitochondrial genome correlate with either schizophrenia and/or bipolar disorder. To date most of these findings were described as polymorphisms occurring in the normal population and did not associate with major psychiatric illness (for a review see Bandelt et al., 2005). The mitochondrial genome is very polymorphic and it is difficult to determine if a newly discovered SNP is pathogenic or merely represents genetic variation. In an effort to perform a more comprehensive analysis, Kazuno et al. (2005) sequenced the entire mitochondrial genome from several patients who had atypical psychosis. Although these authors used a very small sample size and failed to draw any definite conclusion, it will be interesting to see if association studies performed on larger cohorts will uncover disease-related haplotypes.

The ample amount of mitochondrial changes reported in microarray studies and the evidence for an OXPHOS dysfunction in schizophrenia, bipolar disorder and other mental disorders led us to examine if abnormalities in mitochondrial DNA exist in these psychiatric illnesses. We elected to study the mitochondrial common deletion (CD) because (i) it accumulates in various brain regions during

aging together with an OXPHOS decline (Corral-Debrinski et al., 1992), (ii) it plays a major role in classical mitochondrial disorders (Wallace, 1999; Dimauro and DiDonato, 2005), and (iii) it is viewed as an indicator of long-lasting mitochondrial oxidative stress. In addition to quantifying the mitochondrial CD, we also measured the amount of mitochondrial DNA in the frontal cortex to determine if a decrease in mitochondrial DNA is responsible for the global down-regulation of mitochondrial gene transcript expression reported in microarray studies.

Material and methods

Samples

We initially tested if the mitochondrial 5 kb common deletion breakpoint can be amplified reliably from formalin-fixed, paraffin-embedded and frozen human brain sections using two different primer sets. CD quantification by Real-Time-PCR was restricted to the frozen material.

Whole, double-hemispheric, formalin-fixed and paraffin-embedded sections, which allowed the mechanical isolation of distinct brain regions (i.e. hippocampus, thalamus, cingulate gyrus, inferior temporal gyrus, superior temporal gyrus and parietal cortex) from one single section of the same individual, were kindly provided by the Bogerts brain tissue collection at the Department of Psychiatry, Otto-von-Guericke University, Magdeburg, Germany. Following that protocol, the brains were fixed in a 4% paraformaldehyde solution for a period of seven months. After this period, the brainstem and cerebellum were removed, and the brain was cut into two parts. The resulting parts of the whole brain were embedded in paraffin and 10 µm-thick serial sections were generated. A total of 45 brains (15 of each group: control, schizophrenia, bipolar disorder) were used for PCR analysis without prior knowledge of diagnosis. Samples of approximately equal size and corresponding to the above-mentioned regions were delineated on the whole double-hemispheric sections by an experienced neuropathologist (S.W.). They were carefully transferred with a razor blade from the supporting glass tray to an Eppendorf tube. After removal of paraffin with Roticlear (Roth, Germany), DNA was isolated using the Invisorb Spin Tissue Mini Kit from Invitek (Germany) according to the manufacturer's instructions. The DNA was eluted from the Invisorb columns with a buffer volume of 40 µl.

Frozen sections of the frontal cortex (Brodman Area 46), from 60 individuals were kindly provided by the Stanley Medical Research Institute (SMRI) Neuropathology Consortium (for details see Torrey et al., 2000). The sections were received in a coded fashion, and the PCR analysis was performed without knowledge of the diagnosis. The frozen sections were carefully transferred with a razor blade from the standard glass tray into Eppendorf tubes. DNA was isolated using the Invisorb Kit. Three independent DNA isolations were performed from the three frozen sections, which were available from each individual. The slides were transported on dry ice and stored at −80°C until use. In order to minimize DNA degradation after thawing, only a few slides were handled in parallel.

In addition to the brain sections, 10 high quality skeletal muscle DNA samples, prepared from nitrogen-frozen muscle biopsies with the standard phenol/chloroform extraction (Sambrook and Russel, 2001), were used as reference material. The material had been collected from individuals of various ages without any neurological, myological or psychiatric disorders and was stored in the files of the Institute of Neuropathology, Otto-von-Guericke University, Magdeburg, Germany.

In the second part of the study, DNA was obtained from the SMRI microarray collection consisting of 35 control brains and from 35 brains each from patients with schizophrenia and bipolar disorder each. DNA was extracted from the prefrontal cortex (Brodmann area 46). A subset of the samples had to be excluded from the analysis because they exhibited linear amplification curves rather than amplifying exponentially. The reason for this phenomenon is likely to be polymorphisms in the mitochondrial DNA as reported by Kakiuchi et al. (2005). The data presented in this study was finally derived from 29 controls, 30 schizophrenic patients and 25 patients with bipolar disorder resulting in a total N of 84. Each sample was run in triplicate and at least two independent runs were performed. The excluded samples consistently failed to amplify exponentially in each independent run.

Qualitative PCR

For qualitative CD detection, the deletion breakpoint was amplified yielding a fragment of 350 bp.

forward: CCC CTC TAG AGC CCA CTG TA
reverse: GAG TGC TAT AGG CGC TTG TC

Qualitative PCR reactions were carried out in a total reaction volume of 20 µl in a PTC100 thermal cycler (Biozym, Germany). Varying amounts of the isolated DNA were used as templates (see results). In addition to 0.3 units of GoldStar DNA-polymerase (Eurogentec, Belgium) and the appropriate buffer (Eurogentec), the reaction contained 300 nM of the primers, 200 µM of each dNTP and 1.5 mM $MgCl_2$. After an initial denaturation at 93°C for three minutes, 35 PCR cycles followed: 1 min 93°C, 1 min 50°C and 1 min 72°C. The process was completed by a final extension step at 72°C for 7 min. Seven microliters of the reaction product were separated on 0.5 mm thick polyacrylamide gels at 15 W on a multiphor II horizontal electrophoresis device (Pharmacia, Sweden). HaeIII digested Phi-X-174-DNA served as a length standard. The DNA bands were visualized by silver staining, using 3% nitric acid (3 min), 2 g/L $AgNO_3$ (20 min) and a developer, containing 30 g/L Na_2CO_3 and 0.5 ml/L formalin. The reaction was stopped with 10% acetic acid.

Real-Time-PCR

The SYBR Green based Real-Time-PCR was performed on a LightCycler (Roche, USA) with only the 350 bp amplicon measuring the CD and the 400 bp reference sequence of the hypervariable region 2 (HVR2) found in the mitochondrial D-Loop. HVR2 was taken as a reference fragment, since it contains essential elements which are not affected by deletions in replicating mtDNA molecules. Therefore it represents the total amount of mtDNA in a tissue sample. Primer sequences for the 350 bp CD amplicon were the same as described for qualitative PCR (see above) and primers for the HVR2-region were as follows:

forward: CTC TCA CCC TAT TAA CCA CT
reverse: GTT AAA AGT GCA TAC CGC CA

All SYBR Green reactions were carried out in LightCycler capillaries, using the DNA Master SYBR Green I Kit (Roche), 2 µl tissue DNA template, 56°C annealing temperature, 40 cycles and 2.5 mM $MgCl_2$. Dilution series of plasmids containing the CD breakpoint and the HVR2 region were used to calibrate the quantification by the LightCycler software. They were constructed from DNA of a patient with 50% CD in skeletal muscle and from blood samples (HVR2), using the PCR-Script Amp (SK+) vector in Xl-1 blue cells (Stratagene, USA). Both dilution series spanned a range of 10,000-fold dilution in four decadic steps, resulting in standard curves of high linearity ($r > 0.98$). A melting profile was automatically generated after 40 PCR cycles, by slowly melting the double stranded DNA during 10 minutes. A single peak was visible in the plot dF/dT versus T (velocity of fluorescence change versus temperature) for plasmids and frozen tissue sections for both amplified regions. This peak was not present in controls, containing only PCR premix without template DNA. While melting curves of all reactions were inspected, silver-stained polyacrylamide gels (see

above) were used to further check the purity of 10% of the reaction products by an independent method.

While the Real-Time-PCR did not yield any specific products in the formalin-fixed, paraffin-embedded samples, the ratio of CD copy number versus HVR2 copy number in the DNA samples from frozen frontal cortex represented the fraction of mtDNA molecules harboring the CD in this brain region.

In a second phase of the study, the Real-Time-PCR results were extended by a TaqMan assay to ensure maximal specificity. Furthermore a nuclear reference gene (Oncostatin M) was included to allow quantification of total mtDNA. Serially diluted standards were used in the TaqMan assays to determine the relative quantity of CD, mtDNA and genomic DNA. 50 ng of genomic DNA was used for each sample to measure CD and mtDNA amounts. A second, independent set of experiments, used 25 ng of genomic DNA to measure mtDNA and Oncostatin M. All TaqMan reactions were carried out in 384 well plates on an ABI 7900HT instrument. Extracted DNA from the frontal cortex of the SMRI microarray collection was kindly provided by the Stanley Institute. Standard reaction conditions and cycling temperatures recommended by ABI were used and samples and standards were run in triplicate. As above, the HVR2 region was used to normalize the amount of mitochondrial DNA in the samples.

The TaqMan PCR assay primers and probe for the mitochondrial common deletion were:

forward: CTT ACA CTA TTC CTC ATC ACC CAA CTA AAA A
reverse: GGA GTA GAA ACC TGT GAG GAA AGG
MGB probe: CTT TGG CAG CCT AGC ATT

The TaqMan PCR assay primers and probe for the HVR2 region were:

forward: GCT TTC CAC ACA GAC ATC ATA ACA A
reverse: GTT TAA GTG CTG TGG CCA GAA G
MGB probe: AAT TTC CAC CAA ACC CC

The Realtime assay primers and probe specific to the Oncostatin M gene were:

forward: CCT CGG GCT CAG GAA CAA C
reverse: GGC CTT CGT GGG CTC AG
MGB probe: TAC TGC ATG GCC CAG CTG CTG GAC AA

Statistical analysis

Statistical analysis of the Real-Time-PCR data was performed using a multifactorial analysis of variance (ANOVA) and Post-Hoc-Tests for pairwise differences between disease groups and control group.

Confounding variables, grouped according to their scaling properties, were used to assess their influence on the dependent variables. They included age, sex, side of the examined hemisphere, suicide status, smoking at time of death, postmortem interval, brain pH, cerebellar granular cell layer necrosis, rate of death, fixation time of tissue, lifetime antipsychotic intake (in fluphenazine mg. equivalents), data on intake of 1st and 2nd generation antipsychotic generic drugs, mood stabilizer generic drugs, lithium, antidepressant generic drugs, and anticholinergic generic drugs. Other confounding variables will be mentioned in the results section.

Within each of the diagnostic groups, the continuous variables were studied using Pearson and Spearman correlations. If the statistical assumptions of the analysis of covariance were satisfied, such as significant correlations, homogeneity and linearity of the regression lines, then the analysis of covariance was initiated. It is statistically not sensible to perform an analysis of covariance if these assumptions are not satisfied, since the interpretation of the results is highly questionable.

For those ordinal and nominal variables related to drug intake in the patient groups, the control group was used to contrast these variables (obviously the controls by definition had no drug intake) vs. the subjects. Non-parametric statistical procedures, such as contingency tables (Chi-Square), and the Kruskal-Wallis were used to assess the differences if any among the four diagnostic groups.

In order to correlate the rating scale variables with continuous ones, some of the continuous variables were transformed into groups of data to insure that similarly scaled variables were used with the non-parametric correlations.

The software package SPSS for Windows (Release 11.0.1) was used.

Results

Initially, DNA from frozen and formalin-fixed, paraffin-embedded brain sections were extracted since this type of material is easier to obtain and tissue sections allow individual brain structures to be isolated in a more precise manner than a crude tissue dissection. As proof of concept, frozen frontal cortical tissue sections from 10 individuals (age range 25–68 years) were randomly chosen from the SMRI consortium collection, DNA was extracted and the common mitochondrial deletion (CD) was successfully amplified using standard PCR techniques.

Although DNA could be reliably extracted and amplified from frozen tissue sections, working with formalin-fixed, paraffin-embedded tissue sections provided by the Bogerts collection proved to be problematic. The longer amplicon (350 bp) resulted in no visible product on the silver-stained polyacrylamide gels (PAGE), when hippocampus, thalamus, cingulate gyrus, inferior temporal gyrus, superior temporal gyrus or parietal cortex of 10 individuals were tested. Therefore, the remaining 35 individuals were not analyzed with this primer set. The 150 bp amplicon was successfully amplified in a small subset of the 45 cases, resulting in visible PAGE bands, although these bands were usually rather faint, when compared with those amplified from the frozen frontal cortex slices (Fig. 1).

The percentage of CD positive cases from different brain regions was rather low and ranged between 6.9 and 20% of cases. The percentage of CD positive cases for each individual brain regions was as follows: hippocampus: 20% (9 of 45), thalamus: 18% (8 of 45), cingulate gyrus: 8.9% (4 of 45), parietal cortex: 7.8% (2 of 26), inferior temporal gyrus: 6.9% (2 of 29), and superior temporal gyrus: 6.9% (2 of 29). This result suggests an artificial inhibition of

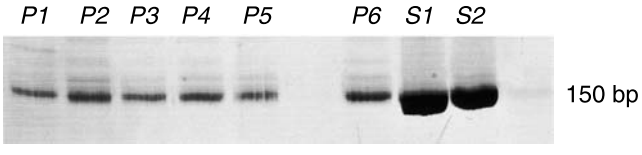

Fig. 1. Comparison of the 150 bp-fragments amplified by qualitative PCR from six formalin-fixed, paraffin-embedded tissue sections (*P1–P6*) and from two frozen brain samples (*S1* and *S2*) of the SMRI collection on a silver stained polyacrylamide gel

PCR amplification by single strand breaks or inhibitors, since the primer set allowed CD detection in 100% of previously tested fresh muscle and brain samples. Therefore, any potential difference between disease and control groups would be likely to reflect a difference in DNA quality rather than a difference in CD abundance. Since the presence of minimal amounts of deleted mtDNA in postmitotic tissues can always be detected by a sensitive PCR assay, e.g. a short amplicon and a high number of PCR cycles, a quantification by Real-Time-PCR or comparable methods is necessary to measure an abnormal accumulation of the CD in diseased tissues.

Nearly all formalin-fixed, paraffin-embedded samples yielded no reaction product with the SYBR Green method. The remaining few samples showed abnormal amplification curves or strongly aberrant melting behaviour. Therefore, formalin-fixed, paraffin-embedded material was completely excluded from the analysis of Real-Time-PCR data. Positive PCR results with the SYBR Green method were obtained from all frozen sections of the frontal cortex (Fig. 2).

Since DNA could be amplified from frozen sections, a SYBR-Green PCR protocol was developed to quantitate the mitochondrial CD in the SMRI brain collection. First, a PCR reaction was established to amplify the CD breakpoint in the tissue sections. A second SYBR-Green reaction was developed to the HVR2 region on the mitochondrial genome in order to quantitate the total amount of mitochondrial DNA present in each sample. Both reactions were run separately on equal amounts of DNA extracted from the same samples. Mitochondrial DNA levels determined by amplifying the HVR2 region were used as an internal control to normalize the mitochondrial CD levels. In other words, the CD abundance in the frontal cortex is expressed as a percentage of deleted mtDNA molecules relative to total mtDNA molecules (HVR2 copies). These primer sets and similar methods have been used extensively for measuring the common mitochondrial deletion in the brain in a variety of different situations (Kakiuchi et al., 2005; Mawrin et al., 2003, 2004a, b; Schoeler et al., 2003), including the quantification of CD loss from hybrid cell cultures (Schoeler et al., 2005). The mean mitochondrial CD values for schizophrenia and for depression are about two times higher compared to the control group, but none of the group differences are significant at the $p < 0.05$ level in a multifactorial analysis of variance (ANOVA) or in pairwise comparisons of groups by Post-Hoc tests.

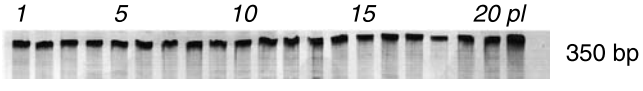

Fig. 2. Silver stained polyacrylamide gel showing 20 samples of frozen frontal cortex and one comigrating cloned plasmid sample (*pl*) after 40 cycles with the SYBR Green method (350 bp amplicon)

The effect of the confounding variables was examined using the data obtained from analysing the frozen brain tissue. Age, sex, hemispheric side, race, postmortem time, unrefrigeration time, refrigeration time, pH, suicide, rate of death, alcohol abuse, drug abuse, use of first generation antipsychotics before death, use of second generation antipsychotics before death, use of antidepressants, mood stabilizers, and lithium before death had no significant effect on the measured parameter. Smoking and the intake of antipsychotics at the time of death had significant effects on the evaluated parameter. Thus, a statistically significant difference ($p = 0.04$) was seen when comparing nonsmokers (mean = 27.32E-05, SEM = 7.13E-05) to smokers (mean = 54.19E-05, SEM = 10.35E-05). When comparing patients who were on antipsychotics at time of death (mean = 60.38E-05, SEM = 15.25E-05) to those who were not on antipsychotics (mean = 23.95E-05, SEM = 6.33E-05), a significant difference was noted ($p = 0.02$).

The common mitochondrial deletion was then measured in a larger set of prefrontal cortex (Brodmann area 46) DNA samples obtained from the SMRI microarray collection. In addition, we developed TaqMan assays to avoid the technical problems associated with SYBR Green Realtime PCR and to see if the TaqMan assay would give corroborating results. Similar to the SYBR Green Realtime assays, a TaqMan assay was designed to the HVR2 region to quantitate the total amount of mitochondrial DNA. A second TaqMan assay targeted the common deletion breakpoint and measured the abundance of the CD in the samples. As before, the amount of CD was normalized using HVR2 levels and the CD amount was expressed as a ratio to the mitochondrial DNA amounts. Figure 3 shows the standard curve for the mitochondrial deletion and the CD amplification plots for the samples examined. Same amount of DNA (50 ng) was used for each sample as well as the HVR2 measurements. CD levels in most of the samples were detected between Ct 25 and 30. Using the TaqMan assay, no statistically significant differences were found for the amount of common mitochondrial deletion between controls and patients with schizophrenia or bipolar disorder, as shown in Fig. 4. In addition, no statistically significant differences were observed for normalized CD levels, age, PMI, brain pH, and refrigeration time. However, female bipolar patients (mean = 2.24E-05, SEM = 7.91E-06) had significantly less CD ($p = 0.03$) then the male patients (mean = 6.61E-05, SEM = 1.39E-05). We also noticed that

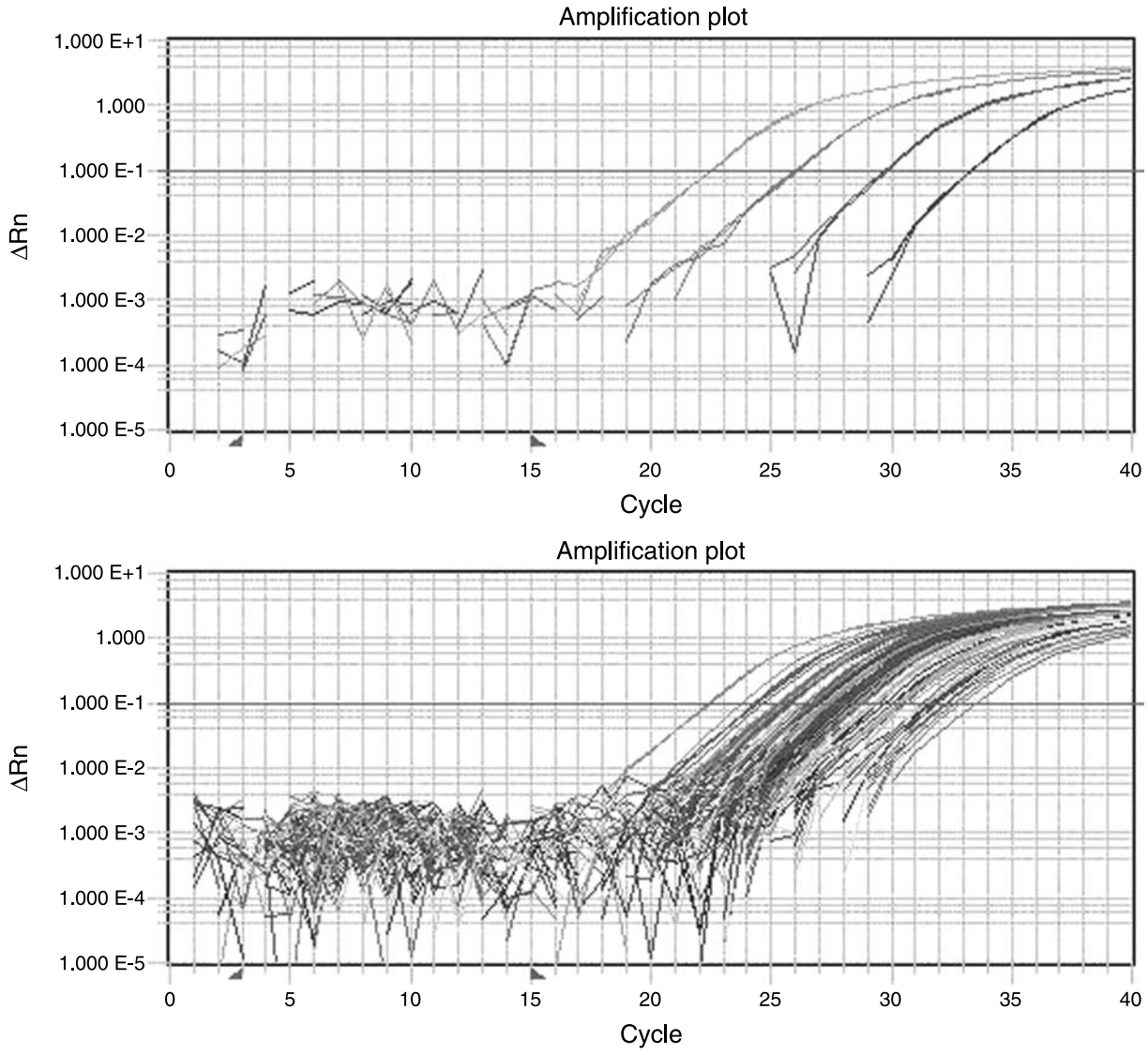

Fig. 3. Taqman PCR assay for measuring the common mitochondrial deletion in post-mortem brain tissue. The top panel shows the amplification plot for the standard curve whereas the bottom panel shows the amplification plot for the post-mortem brain samples. The level of the common mitochondrial deletion in the brain samples is within the linear range of the standard curve

the common mitochondrial deletion differed in cases of antidepressant use (bipolar patients: mean = 7.11E-05, SEM = 1.55E-05 vs. controls: mean = 2.42E-05, SEM = 6.98E-06, $p = 0.02$) and alcohol abuse (bipolar patients: mean = 1.82E-04, SEM = 1.29E-04 vs. control mean = 1.51E-05, SEM = 9.85E-06, $p = 0.04$) in bipolar patients and in case of use of 1st generation antipsychotic drugs in schizophrenic patients (schizophrenia mean = 2.97E-05, SEM = 1.42E-05 vs. control mean = 3.05E-04, SEM = 2.44E-04, $p = 0.02$).

The amount of mitochondrial DNA from the SMRI microarray collection was determined in a set of independent experiments. A TaqMan reaction targeting the single copy number gene oncostatin M (Schoeler et al., 2003) was used to measure the amount of genomic DNA present in samples. The mitochondrial DNA amount (measured using the same TaqMan assay as described above) was normalized to the amount of genomic DNA in the sample and mtDNA abundance in the frontal cortex was expressed as a ratio of mtDNA molecules relative to total genomic DNA molecules. As shown in Fig. 5, the amount of mitochondrial DNA in the frontal cortex did not vary significantly between cases and controls. Based on our statistical analysis age, PMI, brain pH, sex and refrigeration time did not correlate with the reported mitochondrial DNA quantities. However, schizophrenic patients who were using anti-

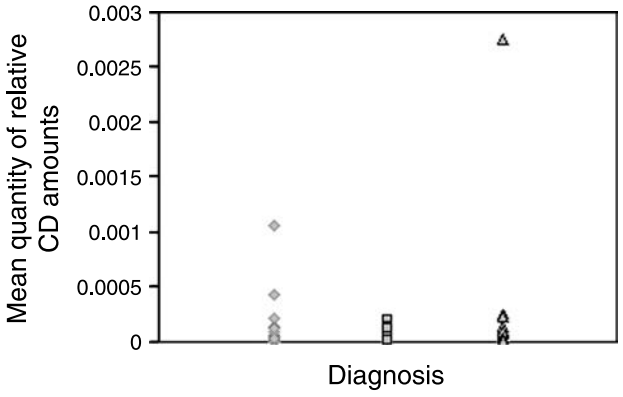

Fig. 4. The common mitochondrial deletion (*CD*) levels measured by TaqMan assay in the prefrontal cortex in the Stanley microarray brain collection. There is no statistically significant difference between any of the diagnosis groups. The values shown represent the percentage of CD/mitochondrial DNA, i.e. CD/HRV2. ◇ Control, □ bipolar, △ schizophrenia

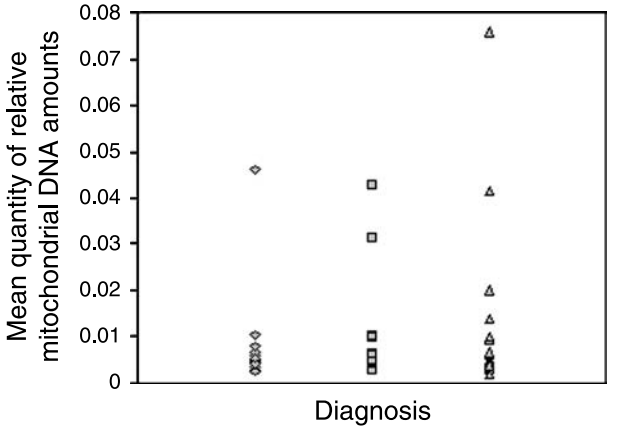

Fig. 5. Mitochondrial DNA levels measured by TaqMan assay in the prefrontal cortex in the Stanley microarray brain collection. There is no statistically significant difference between any of the diagnosis groups. The values shown represent the ratio of mitochondrial DNA/genomic DNA, i.e. HRV2/OSM in arbitrary units. ◇ Control, □ bipolar, △ schizophrenia

psychotics at the time of death had more total mitochondrial DNA level than controls (schizophrenia: mean = 8.45E-03, SEM = 2.91E-03; control: mean = 3.6E-03, SEM = 4.51E-04, $p = 0.04$).

Discussion

In the present study, we developed realtime PCR assays to quantify the common mitochondrial deletion and mitochondrial DNA levels in postmortem brain tissue (prefrontal cortex) obtained from individuals diagnosed with schizophrenia, bipolar disorder, depression and matched controls. We did not see any statistically significant difference amongst diagnosis groups for either of the variables measured. Based on our analysis and the large sample size examined in this study, we conclude that the common mitochondrial deletion and mitochondrial DNA levels are unlikely to contribute to these psychiatric diseases.

In this study we demonstrated that by using DNA isolated from frozen brain tissue sections, it is possible to quantify the mitochondrial common deletion using PCR as long as the tissue samples are of sufficient quality for DNA isolation. The failure to amplify DNA from formalin-fixed, paraffin-embedded samples is attributed to the poor quality of the DNA, i.e. single strand breaks and/or PCR inhibitors. Using frozen brain sections, the developed PCR method was easily adapted for SYBR Green based Real-Time-PCR. The reliability of this method is further verified by the fact that the quantitative results are in accordance with those obtained from normal brains by another research group (Corral-Debrinski et al., 1992), which at that time used a more complicated PCR-based method to quantify the CD in various brain regions of postmortem samples. Although in both studies the inter-individual variability was high, the mean CD abundance across all tested individuals was nearly identical. In the small series of Corral-Debrinsky et al. (1992), the mean CD abundance in cortical areas of persons under 67 years of age was 0.035%, ranging from 0.013 to 0.063% in various cortical regions. Only the values in the cerebellum were much lower and age-independent. The mean CD abundance in the frontal cortex of all persons in our larger series (maximal age 68 years), including all disease groups and controls, was 0.037%. This suggested that the method yielded reliable and comparable quantitative results, provided that the template quality was sufficient.

The mitochondrial DNA region amplified in this study provides a reliable measurement and has been used to analyse mitochondria in other paradigms (Mawrin et al., 2003, 2004a, b; Schoeler et al., 2003). The results reported in this study demonstrate that using postmortem frozen sections from the SMRI collection for real time PCR analysis is feasible at least for quantifying DNA.

To further verify our results, an independent TaqMan PCR assay was designed to measure the mitochondrial common deletion in DNA extracted from pieces of postmortem frontal cortex tissue. The TaqMan assay has the advantage of not suffering from the problems associated with SYBR green real time PCR such as the formation of primer dimers and presence of multiple bands. The common mitochondrial deletion was measured in 60 samples from frozen frontal cortex sections using SYBR green PCR along with DNA from 84 whole frontal cortex tissue samples which were quantified by TaqMan PCR. In both cases,

no statistically significant differences in CD levels between controls and patients were found. Several other studies have examined CD in psychiatric disorders Stine et al. (1993) failed to find any deletions in the mitochondria of 24 individuals with affective disorder using Southern Blot analysis. Kato et al. (1997) re-examined the samples of Stine et al. (1993) using an end point PCR strategy and observed a modest increase in CD in bipolar disorder. However, these findings are based on a very small sample set consisting of only 7 bipolar patients and 9 controls. Furthermore, the tissue used for this study is obtained from the cerebral cortex and is not limited to specific Brodmann areas. Conversely, Cavelier et al. (1995) used a Q-PCR based method to measure CD in 13 schizophrenia brains and 9 controls and failed to detect any differences between the two groups. The data presented in this paper is based on a much larger sample size and the real time PCR assays used in this paper are much more accurate than either Southern Blot analysis or end point PCR. The fact that similar results are obtained by using different starting materials (i.e. slides or tissue), and two completely independent PCR assays further supports our assertion that the common mitochondrial deletion is not likely to play a role in either bipolar disorder or schizophrenia. Because the total number of cases with major depression in this study was small ($N = 15$), such a claim cannot be made for this disorder and further experimentation is required.

Mitochondrial DNA levels were measured as a ratio to genomic DNA which is a standard way of quantifying mtDNA. The single copy number gene oncostatin M was used to get an accurate measurement of genomic DNA copy number in the samples (Bruce et al., 2005). Our results are in agreement with other studies which fail to find a difference in mtDNA levels between cases and controls (Mawrin et al., 2004a). Cavelier et al. (1995) also measured mtDNA copy number and did not detect any differences between schizophrenia and control brains. Similar to the approach reported in this paper, Kakiuchi et al. (2005) measured the ND4 mitochondrial DNA region and assessed the relative amount of mitochondrial DNA to nuclear DNA by quantitating the single copy gene RNAseP in the samples. Although, they amplified a different region on the mitochondrial genome and used a different gene to measure genomic DNA levels, Kakiuchi et al. (2005) also did not find mitochondrial DNA levels to vary between cases and controls. Since our data, which are based on a completely independent set of measurements, corroborate the findings of Kakiuchi et al. (2005), changes in mitochondrial DNA levels are also unlikely to contribute to the pathophysiology of either schizophrenia or bipolar disorder.

Since brains from the SMRI collection were used as the starting material for this paper and since many of the microarray studies which report a decrease in mitochondrial transcripts used the same starting material (Prabakaran et al., 2004; Altar et al., 2005; Iwamoto et al., 2005), sampling error or differences in cohorts can be ruled out as a possible reason for not detecting mtDNA changes in this study. Novel abnormalities or mutations in mtDNA may be responsible for the reported down-regulation of mitochondrial transcripts in psychiatric disorders. However, these mtDNA abnormalities are likely to be subtle since sequencing of the whole mitochondrial genome in psychotic patients did not reveal any major deletions or rearrangements in mtDNA which associate with psychosis (Kazuno et al., 2005). Several SNP's were discovered in the mtDNA of psychiatric patients but it is not clear if these SNP's are related to disease or simply represent polymorphic loci. In fact, the down-regulation of mitochondrial gene expression reported in microarray experiments (Konradi et al., 2004; Prabakaran et al., 2004; Altar et al., 2005; Iwamoto et al., 2005) may not even be related to the mtDNA sequence. A combination of regulatory factors acting at the transcriptional, or the post-transcriptional levels may be responsible for the observed decrease in mitochondrial RNA levels. Decreased activity of respiratory chain complexes (e.g. COX) may alternatively be explained by nuclear DNA encoded regulatory factors, including proteins which assist assembly and maintenance of respiratory chain complexes in the inner mitochondrial membrane (e.g. SURF1 assisting COX). Additionally, mtDNA levels maybe altered or the common mitochondrial deletion may exist in brain regions other than the frontal cortex. Furthermore, cell type specific changes in mtDNA cannot be determined by the global amplification technique used in this paper. Significant changes in mtDNA occurring in a small number of cells, i.e. only GABAergic or only glutamatergic, can easily go undetected via Real-Time-PCR which amplifies everything that is in the frontal cortex. Mitochondrial DNA needs to be measured from laser microdissected cells (a strategy similar to the one employed by Altar et al. (2005)) in order to clarify this matter. A combination of sophisticated long PCR techniques and micro dissection was recently used to prove a dramatic accumulation of deleted mtDNA in the dopaminergic neurons of the substantia nigra (Bender et al., 2006; Kraytsberg et al., 2006). However, developing this sort of an assay is not a trivial matter and was beyond the scope of this article.

Several recent papers (Vawter et al., 2006; Tomita et al., 2004; Li et al., 2004) have demonstrated that agonal factors and brain pH may influence mitochondrial RNA

levels and have raised concerns about the validity of mitochondrial gene expression differences described in microarray studies. However, the findings of these reports can not be interpreted to mean that mitochondrial dysfunction does not exist in psychiatric disorders. Data from functional brain imaging studies (Volz et al., 1997; Kato et al., 1993) suggests that aberrant mitochondrial functioning is involved in the pathophysiology of these diseases. Elevation of gray matter lactate levels in unmedicated bipolar patients provides further evidence for this hypothesis (Dager et al., 2004). Based on these *in vivo* studies, we conclude that the presence of confounding factors which influence mitochondrial RNA measurements do not exclude the possibility that mitochondrial dysfunction is present in psychiatric disorders. Note that we did not find any correlation between brain pH and either mtDNA quantity or the common mitochondrial deletion.

Finally, in our statistical analysis we observed that female bipolar patients had significantly more CD then the male patients. We do not have an explanation for this observation and further experiments need to be conducted to verify it. This difference was not seen in the SYBR green assays. In addition, our observations that the use of antidepressants and alcohol in bipolar patients and the use of 1st generation antipsychotic drugs in patients with schizophrenia correlated with the common mitochondrial deletion are interesting but need to be verified either by using animal models or by creating cohorts which are designed specifically to investigate these variables.

With respect to mitochondrial oxidative stress and dysfunction, psychiatric drugs, might represent an additional problem. Prince et al. (1997) showed, that the significant COX-deficiency in the frontal cortex of schizophrenic patients could not be explained by haloperidol or fluphenazine treatment. Nevertheless, they found at least some discrete, adverse effects on mitochondrial respiratory chain. These results could be compatible with a partial inhibition of the respiratory chain complexes, which might result in enhanced superoxide production. Thus, a potential influence of drug treatment on CD abundance would have to be analyzed, in addition.

At present, the role of mitochondrial dysfunction and oxidative stress in psychiatric disorders remains unresolved. Mitochondria are novel and intriguing therapeutic targets but more research has to be conducted in order to firmly establish a link between mitochondrial deficits and schizophrenia, bipolar disorder and other psychiatric diseases.

Acknowledgements

The authors thank Theodore and Vada Stanley as well as the Stanley Medical Research Institute for financial support of the present study, the staff of the SMRI brain bank for providing the tissue, Ms. Ines Schellhase for skillfull technical support, and Dr. E. Fuller Torrey for contributing the clinical data.

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