Mitochondrial Mutations in Subjects with Psychiatric Disorders

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Abstract

A considerable body of evidence supports the role of mitochondrial dysfunction in psychiatric disorders and mitochondrial DNA (mtDNA) mutations are known to alter brain energy metabolism, neurotransmission, and cause neurodegenerative disorders. Genetic studies focusing on common nuclear genome variants associated with these disorders have produced genome wide significant results but those studies have not directly studied mtDNA variants. The purpose of this study is to investigate, using next generation sequencing, the involvement of mtDNA variation in bipolar disorder, schizophrenia, major depressive disorder, and methamphetamine use. MtDNA extracted from multiple brain regions and blood were sequenced (121 mtDNA samples with an average of 8,800x coverage) and compared to an electronic database containing 26,850 mtDNA genomes. We confirmed novel and rare variants, and confirmed next generation sequencing error hotspots by traditional sequencing and genotyping methods. We observed a significant increase of non-synonymous mutations found in individuals with schizophrenia. Novel and rare non-synonymous mutations were found in psychiatric cases in mtDNA genes: ND6, ATP6, CYTB, and ND2. We also observed mtDNA heteroplasmy in brain at a locus previously associated with schizophrenia (T16519C). Large differences in heteroplasmy levels across brain regions within subjects suggest that somatic mutations accumulate differentially in brain regions. Finally, multiplasmy, a heteroplasmic measure of repeat length, was observed in brain from selective cases at a higher frequency than controls. These results offer support for increased rates of mtDNA substitutions in schizophrenia shown in our prior results. The variable levels of heteroplasmic/multiplasmic somatic mutations that occur in brain may be indicators of genetic instability in mtDNA.
Introduction

The mitochondrial hypothesis of psychiatric disorders derives from evidence of energy metabolism alterations, high prevalence of affective disorders in patients with mitochondrial disorders, and from increased maternal heritability [1]. Cross-sectional risk studies have revealed a significantly higher risk for schizophrenia in relatives who shared mitochondrial DNA (mtDNA) with a schizophrenia patient [2]. However, studies concentrating on major mtDNA haplogroups have failed to reveal clear differences between these major haplogroups in terms of risk to develop psychiatric disorders [3–7]. Recent studies have also suggested that variants in mtDNA can contribute to the risk to develop major depressive disorder (MDD), bipolar disorder (BD) and schizophrenia (SZ) [8–13]. Additionally, some patients with mitochondrial disorders caused by known mtDNA mutations often present psychiatric symptoms [14–16], suggesting a major role of mtDNA mutations in the predisposition to psychiatric disorders. Incidentally, in a large population analysis, common mtDNA variants have been shown to also increase the risk of many seemingly unrelated diseases, some affecting the brain such as ischemic stroke, multiple sclerosis and Parkinson’s disease [13].

The mitochondrial DNA (mtDNA) is a 16.6 kb circular molecule maternally transmitted and located inside the mitochondrion. The main role of mitochondria is to produce energy through oxidative phosphorylation (OXPHOS). The mtDNA genome encodes 13 OXPHOS proteins, 22 tRNAs, and 12S and 16S rRNA genes. Because each cell contains between 100 to 1000 mitochondria, and each mitochondrion contains a variable number of mtDNA molecules [17, 18], mtDNA mutations can be homoplasmic (present in all copies of the mtDNA genome) or heteroplasmic, with mutations only present in a fraction of the mtDNA molecules. In the past, cloning and Sanger sequencing have been used to investigate heteroplasmy levels, but recent next-generation sequencing (NGS) advancements now allow the study of mtDNA variation with sufficient coverage to uncover heteroplasmy [9, 19, 20].

Genetic predisposition for psychiatric disorders has been extensively studied but few candidate gene variants have been validated across cohorts. However, most of these studies have focused on nuclear genes instead of mtDNA variants. The mitochondrial genome is particularly sensitive to oxidative stress and tends to accumulate somatic mutations with age, particularly in high energy demanding regions such as the brain. Chronic methamphetamine (METH) use is also associated with increased oxidative stress and mitochondrial dysfunction [21]. We therefore included a group of METH users to investigate the chronic effects of this drug on somatic mutations in METH susceptible regions of the brain.

Prior studies, using NGS, have shown an increase in somatic homoplasmic and heteroplasmic mtDNA mutations in cancer [22], cardiomyopathy [23], and aging [24, 25]. We recently used NGS to investigate the involvement of mtDNA somatic homoplasmic mutations in a small sample of brains from patients with psychiatric disorders [9].

In this study, we investigated the involvement of homoplasmic, multiplasmic and heteroplasmic variation in mtDNA from 69 subjects, using NGS on 11 brain regions and blood samples from patients with psychiatric conditions and normal controls. Our working hypothesis was that a greater number of mtDNA mutations would occur in cases compared to controls. We also hypothesized that somatic mutations can appear in some brain regions and accumulate to a deleterious level and play a role in the pathophysiology of psychiatric disorders. Brain tissue is a unique resource to investigate the occurrence of heteroplasmic mutations not necessarily present in peripheral tissues such as blood.
Results

We analyzed 121 complete mtDNA sequences from 69 subjects, including samples from several brain regions and from blood for three subjects (S1 and S2 Tables). All 121 mtDNA sequences passed stringent quality control and were deposited at NCBI (http://www.ncbi.nlm.nih.gov/) accession numbers KC257284-KC257404. Despite differences in overall coverage, reflecting differences between the two platforms efficiency and our multiplexing, we did not observe major differences in the variants reported by the two Illumina platforms (GAII (cohort 1) and HiSeq (cohort 2)). GAII produced an average coverage for the variants of 3,766 (min = 100, max = 15,620) and the HiSeq platform an average of 9,775 (min = 1,114, max = 107,710), with a combined overall average coverage of 8,850.

Mitochondrial DNA variation

Of the 3,670 mtDNA variants across the 121 samples, the majority were homoplasmic (3177) and 493 were heteroplasmic. The majority of heteroplasmic variants were C>T or A>G transitions, consistent with the expected transition to transversion rate [26]. Consensus mtDNA sequences of the 121 samples were used to build a phylogenetic tree (S1 Fig) following established PhyloTree topology and haplogroup nomenclature24. Subjects were distributed across diverse haplogroups with no clear clustering of diagnosis, suggesting that there is no specific increase in the predisposition to psychiatric disorders in mitochondria haplogroups (S1 Fig). Given the perfect agreement of consensus sequences across brain regions and blood, we decided to focus on the DLPFC data to compare subjects based on diagnosis. We observed a total of 1748 sequence variants in the DLPFC from 63 unique subjects, but many of them were haplogroup specific and reflected divergence from the mitochondrial revised Cambridge Reference Sequence (rCRS; GenBank accession number NC_012920)[27]. The rCRS, often used as the reference, is a useful tool to compare mitochondrial genomes but does not represent the most common haplotype or an ancestral haplotype, it is simply one haplotype. One subject with schizophrenia, for instance, was identical to the rCRS, while a normal control of African American ancestry carried a large number of divergent loci compared to the rCRS. We therefore excluded the major haplogroup defining variants to explore the specific involvement of mitochondrial variation in psychiatric disorders. A total of 1,175 variants in the DLPFC were further investigated, 984 were homoplasmic (S3 Table) and mainly located in the hypervariable region of the mtDNA, and 191 were heteroplasmic (see section regarding heteroplasmic variation).

Homoplasmic variants

Of the 984 homoplasmic variants, 141 were located within genes and were non-synonymous, therefore potentially functional (S4 Table). Comparison of variants only observed in cases or only observed in controls revealed 49 non-synonymous variants (37 loci) only observed among 43 cases (Table 1) versus 12 non-synonymous variants only present in the 20 controls. Of these 37 loci, a total of 8 were predicted using Polyphen as being possibly/probably damaging mutations. There were 80 shared variants between cases and controls. The ratio of the number of non-synonymous mutations to genomes sequenced revealed a significantly higher (p = 0.024) number of mutations in SZ (1.57) versus controls (0.55) (Table 2). We next tested whether the distribution of the total number of non-synonymous mutations in cases was different compared to controls and found a non-significant trend for five or more non-synonymous mutations in cases compared to controls (p = 0.068, S5 Table). We found six homoplasmic non-synonymous mutations that have not been previously reported in MITOMAP, mtDB, or PhyloTree (Table 3). Two of these mutations were only found in an online ancestry database
Table 1. Non-synonymous homoplasmic substitutions at 37 loci were found only present in cases but not in control DLPFC samples.

| Position | Gene | BD | MDD | SZ | Total | Reference | Observed | AA | Change | Prediction | Haplogroup | Mitomap (N) | SwissProt |
|----------|------|----|-----|----|-------|-----------|----------|-----|---------|------------|------------|-------------|-----------|
| 3509*    | ND1  | 1  | 1   | T  | C     | Ile68Thr  | benign   | H61 |          |            |            |             | P03886    |
| 3796     | ND1  | 1  | 1   | 2  | A     | Thr164Ala | benign   | H1b1, H1b1 | 157 |         |            |            |             | P03886    |
| 3992     | ND1  | 2  | 2   | C  | T     | Thr229Met | benign   | H4a1a1a, H2a2a1 | 212 |         |            |            |             | P03886    |
| 4024     | ND1  | 2  | 2   | A  | G     | Thr240Ala | benign   | H4a1a1a, H2a2a1 | 166 |         |            |            |             | P03886    |
| 4025     | ND1  | 1  | 1   | C  | T     | Thr240Met | benign   | H3h  |          |            |            |             | P03886    |
| 4561     | ND2  | 1  | 1   | T  | C     | Val31Ala  | benign   | K2a10 |          |            |            |             | P03891    |
| 4732     | ND2  | 1  | 1   | A  | G     | Asn88Ser  | benign   | U5b2a1a1b | 198 |         |            |            |             | P03891    |
| 4824     | ND2  | 1  | 1   | 2  | A     | Thr119Ala | possibly damaging | A2d, A2p | 746 |         |            |            |             | P03891    |
| 4924*    | ND2  | 1  | 1   | G  | C     | Ser152Thr | benign   | U5a1d2b | 7   |         |            |            |             | P03891    |
| 5073*    | ND2  | 1  | 1   | A  | G     | Ile202Val | benign   | K1b2b | 1   |         |            |            |             | P03891    |
| 5277     | ND2  | 1  | 1   | T  | C     | Phe270Leu | benign   | A2p  | 75  |         |            |             |             | P03891    |
| 5913     | COX1 | 1  | 1   | G  | A     | Asp44Asn  | benign   | K1b2b | 191 |         |            |            |             | P00395    |
| 6366     | COX1 | 1  | 1   | G  | A     | Val155ile | benign   | A2d  | 98  |         |            |             |             | P00395    |
| 6480     | COX1 | 1  | 1   | G  | A     | Val193ile | benign   | I2d  | 70  |         |            |             |             | P00395    |
| 8108     | COX2 | 1  | 1   | A  | G     | Ile175Val | benign   | A2d  | 45  |         |            |             |             | P00403    |
| 8463*    | ATP8 | 1  | 1   | A  | G     | Tyr33Cys  | probably damaging | X2c1b | 8   |         |            |            |             | P03928    |
| 8519     | ATP8 | 1  | 1   | G  | A     | Glu52Lys  | possibly damaging | I4a   | 63  |         |            |            |             | P03928    |
| 8794     | ATP6 | 1  | 1   | 2  | C     | His90Tyr  | benign   | A2d, A2p | 726 |         |            |            |             | P00846    |
| 8843     | ATP6 | 1  | 1   | T  | C     | Ile106Thr | possibly damaging | T2b4  | 119 |         |            |            |             | P00846    |
| 9055     | ATP6 | 2  | 1   | 3  | G     | Ala177Thr | possibly damaging | K2a10, K1b2b, K1a3a | 1401 |         |            |            |             | P00846    |
| 9160*    | ATP6 | 1  | 1   | T  | C     | Tyr212His | probably damaging | A2d  | 1   |         |            |            |             | P00846    |
| 9210     | COX3 | 1  | 1   | A  | G     | Thr2Ala   | benign   | H1ag  | 33  |         |            |            |             | P00414    |
| 11016    | ND4  | 1  | 1   | G  | A     | Ser76Asn  | benign   | H48   | 184 |         |            |            |             | P03905    |
| 11204    | ND4  | 1  | 1   | T  | C     | Phe149Leu | benign   | H1    | 100 |         |            |            |             | P03905    |
| 12346    | ND5  | 1  | 1   | C  | T     | His47Tyr  | unknown  | U2e1c | 168 |         |            |            |             | P03915    |
| 12397    | ND5  | 1  | 1   | A  | G     | Thr21Ala  | unknown  | X2a2  | 134 |         |            |            |             | P03915    |
| 12811    | ND5  | 1  | 1   | T  | C     | Tyr159His | benign   | H3h   | 214 |         |            |            |             | P03915    |
| 13117    | ND5  | 1  | 1   | A  | G     | Ile261Val | possibly damaging | K1a3a | 47  |         |            |            |             | P03915    |
| 13637    | ND5  | 1  | 1   | A  | G     | Gly342Arg | benign   | U5b2a1a1b | 243 |         |            |            |             | P03915    |
| 13708    | ND5  | 1  | 1   | 2  | G     | Ala458Thr | benign   | J1c2o, U5b2a1a1b | 1912 |         |            |            |             | P03915    |
| 14110    | ND5  | 1  | 1   | T  | C     | Phe592Leu | benign   | H1    | 247 |         |            |            |             | P03915    |
| 14280*   | ND6  | 1  | 1   | A  | C     | Ser132Ala | benign   | U2e1c | 2   |         |            |            |             | P03923    |
| 14502    | ND6  | 1  | 1   | T  | C     | Ile58Val  | benign   | X2a2  | 111 |         |            |            |             | P03923    |
| 14582    | ND6  | 2  | 2   | A  | G     | Val31Ala  | benign   | H4a1a1a, H2a2a1 | 164 |         |            |            |             | P03923    |
| 14798    | CYTB | 1  | 2   | 1  | 4     | Phe18Leu  | benign   | J1c2o, K2a10, K1b2b, K1a3a | 2114 |         |            |            |             | P00156    |
| 14982*   | CYTB | 1  | 1   | T  | C     | Ile79Thr  | possibly damaging | V16   | 2   |         |            |            |             | P00156    |
| 15431    | CYTB | 1  | 1   | G  | A     | Ala229Thr | benign   | I3b   | 326 |         |            |            |             | P00156    |

The observed haplogroups are randomly distributed. The effect of the amino acid substitutions was determined using Polyphen, damaging mutations are shown in bold.

* Frequency corresponds to less than 0.1% of 26,850 human mtDNA sequences with size greater than 15.4 kbp collected from GenBank on 25 June 2014.

# Mutations were found in Mitomap (accessed June 2014) as a result of our NCBI data deposit.

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but were not present in the PhyloTree database containing 16,810 mtDNA sequences as of September 2012 [28]. Of these six non-synonymous novel and rare variants, four were located in ND6, ATP6, CYTB and ND2, and were only observed in cases while several mutations located in ATP6 (Tyr212His, Asn39Thr and Met140Thr) but present both in controls and cases, which were predicted to be damaging by Polyphen.

Recently, whole genome sequencing studies have shown increased mutation rates in schizophrenia but only examined nuclear genes [29, 30]. In the current study, we have focused on the occurrence of nonsynonymous variants in mtDNA as another distinct possible genetic predisposition to schizophrenia.

Several mutations in ribosomal RNA (rRNA) were also observed only in cases not in controls (S6 Table in gray). The 16S rRNA had 10 variants only observed in cases and the 12S rRNA had 6 variants that were only observed in cases. The z-score difference between the number of unique rRNA variants for controls and schizophrenia was not significant (p = 0.15). Two of these rRNA variants were rare mutations present only in an MDD subject (C1601T) and in a BD subject (T1861C). After querying multiple online databases these mutations were found via Ian Logan’s website in accessions HM238202 (Philippines; haplogroup B4a1a) and JN872374 (Italy; haplogroup U1a3). The C1601T mutation occurs at the 3’ terminus of the 12S mtDNA rRNA and does not appear to pose functional effects, while the T1861C mutation occurs at a non-complementary bridge, that increases complementarities in that region.

The number of non-synonymous mutations found only in cases (and not controls) is shown on each row for each disorder. The ratio of the number of mutations / subject was calculated for each group, and a z-score for the difference of the observed for each group compared to the entire mean was calculated. There was a significant increase in non-synonymous mutations (p = 0.024, two tailed z-score test) in persons with SZ compared to controls.

The number of non-synonymous mutations shown in S4 Table, was extracted when present only in controls and not cases with psychiatric disorders, and is shown in the row for control subjects.

| Subjects | Mutations | Mutations/subject | z-score |
|----------|-----------|-------------------|---------|
| Controls | 20        | 11                | 0.55    | -0.990 |
| BD       | 14        | 14                | 1.00    | -0.056 |
| MDD      | 15        | 13                | 0.87    | -0.333 |
| SZ       | 14        | 22                | 1.57    | 1.380  |

The effects of the amino acid substitutions were determined using Polyphen.

Table 3. Novel-rare NS mutations observed in 65 DLPFC brain samples and confirmed by Sanger sequencing, the D-loop mutation is non-coding.

| Gene | Mutation | Coverage | Amino Acid Change | Age | Gender | Axis | Haplogroup | Status |
|------|----------|----------|-------------------|-----|--------|------|------------|--------|
| ATP6 | T8945C* | 2820     | Met140Thr-damaging | 55  | M      | C    | H1         | Rare   |
| ATP6 | A8642C | 2572     | Asn39Thr-damaging  | 54  | M      | C    | I1         | Novel  |
| ATP6 | T9160C  | 28247    | Tyr212His-damaging | 50  | M      | BD   | A2d        | Novel  |
| CYTB | T14982C* | 12465   | Ile79Thr-moderate  | 41  | F      | MDD | V16        | Rare   |
| ND6  | A14280C | 1113     | Ser132Ala-benign   | 36  | M      | SZ   | U2e1c      | Novel  |
| ND2  | A5073G | 20128    | Ile202Val-benign   | 40  | F      | SZ   | K1b2b      | Novel  |
| D-loop | T16178G | 22475   | -                  | 35  | M      | SZ   | A2p        | Novel  |

The effect of the amino acid substitutions was determined using Polyphen.

*Once in submitted online mtDNA sequencing data.

1 T8945C was previously reported by our group [9].

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Heteroplasmic variation in mtDNA

We observed a total of 114 heteroplasmic variants in 69 unique mtDNA genomes. Heteroplasmic mutations were defined as any variant for which the major allele was <95% of the total of the observed alleles and a minimum coverage of 200 reads. As expected, the heteroplasmic variants were mainly clustered within the hypervariable region (S7 Table). No clear over-representation of heteroplasmic mutations was observed in cases versus controls (except for the multiplasmy described below). Four heteroplasmic variants with high heteroplasmy and two multiplasmic variants were successfully validated by Sanger sequencing, confirming the reliability of next generation sequencing and of our quality control criteria for the investigation of heteroplasmic mtDNA variation (Table 4).

Table 4. Heteroplasmy and multiplasmy in mtDNA.

| Subject | Haplogroup | Region | Diagnosis | Gender | Age | Position | Reference | Alleles | Heteroplasmy | Gene | Status |
|---------|------------|--------|-----------|--------|-----|----------|-----------|---------|--------------|------|--------|
| Heteroplasmy | C-58 | L1c3b2 | All* | C | F | 58 | 11696* | G | G/A | 24.8–28.3% | ND4 | Known |
| C-58 | L1c3b2 | All* | C | F | 58 | 16086 | T | T/C | 67.4–93.7% | D-loop | Known |
| M-18 | N1a1a1b2 | DLPFC | MDD | M | 35 | 16086 | T | T/C | 85.5% | D-loop | Known |
| M-18 | N1a1a1b2 | DLPFC | MDD | M | 35 | 16261 | C | C/T | 70.9% | D-loop | Known |
| S-111 | H56 | DLPFC | SZ | M | 47 | 16519 | T | T/C | 18.9% | D-loop | Novel |
| D-84 | H69 | DLPFC | Meth | F | 34 | 16519 | T | T/C | 90.0% | D-loop | Novel |

Multiplasmy

| Multiplasmy | C-25 | H5d | All* | C | M | 64 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| C-58 | L1c3b2 | All* | C | F | 58 | 514 | T | T/C | 67.4–93.7% | D-loop | Known |
| C-83 | U5b1e1 | DLPFC | C | M | 44 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| B-71 | X2a2 | DLPFC | BD | F | 70 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| B-76 | I3b | DLPFC | BD | M | 39 | 514 | (CA)5 | (CA)3,6,7 | D-loop | Known |
| B-78 | A2d | DLPFC | BD | M | 50 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| B-79 | H1 | DLPFC | BD | M | 43 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| D-85 | A2c | SN | Meth | M | 35 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| D-86 | H2a2b1a | SN | Meth | F | 39 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| M-96 | I4a | DLPFC | MDD | M | 61 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| M-97 | H1b1 | DLPFC | MDD | F | 63 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| M-98 | HV | DLPFC | MDD | F | 41 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| S-110 | U5b2a1a1b | DLPFC | SZ | F | 41 | 514 | (CA)5 | (CA)5,6 | D-loop | Known |
| S-106 | H4a1a1a | DLPFC | SZ | M | 59 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| S-107 | H2a2a1 | DLPFC | SZ | M | 45 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| S-112 | A2p | DLPFC | SZ | M | 35 | 514 | (CA)5 | (CA)4,5 | D-loop | Known |
| S-114 | K1b2b | DLPFC | SZ | F | 40 | 514 | (CA)5 | (CA)5,6,7 | D-loop | Known |
| C-83E | U5b1e1 | DLPFC | C | M | 44 | D16189 | (C)10 | (C)10,11 | D-loop | Known |
| C-90E | B2g | DLPFC | C | F | 32 | D16189 | (C)10 | (C)10,11 | D-loop | Known |
| C-89E | H1ap1 | DLPFC | C | M | 68 | D16189 | (C)10 | (C)10,11 | D-loop | Known |
| B-77E | H1b | DLPFC | BD | M | 50 | D16189 | (C)10 | (C)10,11 | D-loop | Known |
| B-71E | X2a2 | DLPFC | BD | F | 70 | D16189 | (C)10 | (C)10,11 | D-loop | Known |
| M-97E | H1b1 | DLPFC | MDD | F | 63 | D16189 | (C)10 | (C)10,11 | D-loop | Known |

The heteroplasmy was calculated from NGS reads and confirmed by Sanger sequencing (Fig 1B). Multiplasmic length polymorphisms were observed in the D-loop region and showed a trend for over-representation of cases at the 514 (CA)6 and the D16189 loci. Multiplasmy was observed about 1.5 times more frequently in cases. Note the tri-allelic multiplasmy results for two subjects (B-76 and S-114) showing 5, 6, and 7 repeat lengths shown in bold. Heteroplasmy/multiplasmy at all these six loci were also confirmed by Sanger sequencing.

*multiplasmy occurred in all brain regions sequenced.

#Val313Ile.

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We further studied the heteroplasmy of T16519C because of the prior association of this SNP in a GAIN/WTCCC2 association analysis in SZ and BD [9]. We confirmed a heteroplasmic T>C substitution at position 16519 of the mitochondrial genome by allele specific PCR using locked nucleic acid primers (LNA-primers) as well as by direct sequencing. Low levels of T16519C heteroplasmy calculated using NGS (~10%) were confirmed by Sanger sequencing as shown in Fig 1A. Heteroplasmy levels ranged from 1.74%, 18.9%, and 90% calculated from Illumina NGS results. Interestingly, within the same METH subject, we observed, by NGS and Sanger, homoplasmy in one brain region (100% C in the SN (sample D-84J) and heteroplasmy in the DLPFC (sample D-84E) with 90% C and 10% T (Fig 1A). Another example of variable levels of heteroplasmy was observed across all brain regions for a control subject at mt16086 (Fig 1B). Levels ranged from 6.3% in the NACC to 32.5% in the THAL (Fig 1B). These findings clearly demonstrate how heteroplasmic mutations can vary between brain regions of the same individual. Interestingly, sample D-84 was a METH user but overall we did not observe an increase in somatic mutations associated with METH.

**Multiplasmy**

Multiplasmy is a heteroplasmic variant occurring at a variable length repeat locus. As an example, a repeat of ‘CA’ might be a variable length of 5, 6, and 7 repeats. Thus, a single mtDNA molecule could have one of these three repeat lengths, and taken together in one individual, this locus could have all three repeat lengths. Heteroplasmic deletion/insertion polymorphisms analysis showed a high number of multiplasmic subjects in loci previously known to be particularly hypervariable. Two of these were the poly-cytosine tracts of the hypervariable region, the D310 poly-C tract (CCCCCTCCCCC from position 303 to 316) and the D16189 poly-C tract...
Multiplasmy was also observed at a locus known to be a highly variable dinucleotide repeat \((\text{CA})_n\) beginning at position 514 of the D-loop. \(\text{CA}_5\) is the rCRS sequence and we observed 4, 5, 6, and 7 CA repeats (Fig 1B, Table 4).

Multiplasmy was observed about 1.5 times more frequently in cases (Table 4). Some subjects (B-76 and S-114) even show tri-allelic multiplasmy with a combination of 5, 6, and 7 CA repeats. The ratio of 514 CA deletions across brain regions was variable (S2 Fig), and deletions, when present, were found across all 10 brain regions.

**Blood-brain comparisons**

Homoplasmic and heteroplasmic mtDNA variants were compared between blood and 11 brain regions using samples from three control subjects. Although this is too small of a sample to draw any definitive conclusion, there was perfect concordance between the homoplasmic variants found in the 11 brain regions and blood for these three subjects (data not shown), suggesting that blood might be a useful surrogate for the study of homoplasmic mtDNA variation of germ line origin. However, we observed 5 loci with subtle differences for heteroplasmic variants that were present at various levels in brain tissue but undetectable in blood using the 5% cutoff used in the present study (S8 Table). Three of these loci (2487, 5755, and 13706) were not present in our database search for reported variants.

**Discussion**

We observed several novel and rare mtDNA coding homoplasmic mutations in key genes (ND6, ATP6, CYTB, and ND2). Four novel non-synonymous homoplasmic mutations were

![C16086T](C16086T.png)

**Fig 2. Heteroplasmy levels at position 16086 across 11 brain regions for the same subject.**

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(CCCCTCCCC from 16184 to 16193)[29, 30]. Multiplasmy was also observed at a locus known to be a highly variable dinucleotide repeat \((\text{CA})_n\) beginning at position 514 of the D-loop. \(\text{CA}_5\) is the rCRS sequence and we observed 4, 5, 6, and 7 CA repeats (Fig 1B, Table 4). Multiplasmy was observed about 1.5 times more frequently in cases (Table 4). Some subjects (B-76 and S-114) even show tri-allelic multiplasmy with a combination of 5, 6, and 7 CA repeats. The ratio of 514 CA deletions across brain regions was variable (S2 Fig), and deletions, when present, were found across all 10 brain regions.
We validated in different coding regions, three of which were present only in cases and not in controls. There was an excess of non-synonymous homoplasmic mutations found in schizophrenia, but not controls. We also confirmed heteroplasmy at a locus in the D-loop region (T16519C), that we previously reported as being associated with SZ [9]. Excess multiplasmy in cases at the 16189 poly-C tract and at the 514 (CA)_n repeat region was also observed, as well as single SZ and BD cases showing striking tri-allelic multiplasmy in brain.

Evidence of genomic instability in the form of somatic variation at heteroplasmic and multiplasmic loci, and novel and rare variants, are particularly interesting in light of recent studies using NGS that showed an excess of novel and rare functional variants in the nuclear genome in different populations [31] and their potential role in complex traits and drug response [32]. A recent study explored the presence of somatic mutations in the aging human brain and showed an accumulation of deletions and single nucleotide variants with age specially in the non-coding hyper-variable region [33], consistent with our findings of somatic heteroplasmic mutations in the adult human brain. The rare and novel coding variants that we found, and the additional non-synonymous mutations in the mtDNA of psychiatric cases, could also support abnormal energy metabolism seen using Magnetic Resonance Spectroscopy (MRS). In general, studies of patients with BD, MDD, and SZ have shown altered energy metabolism in brain [34, 35]. Potential treatment responders to antidepressants sometimes show alterations in MRS profile of energy metabolites [36]. In an animal model of depression there was an altered metabolic profile that was restored to control levels following antidepressant treatment [37]. In view of these evidences, it would be interesting to test the effect of mtDNA variation on energy metabolism in peripheral samples from psychiatric patients.

MtDNA can be methylated [38] suggesting an additional control of mitochondrial transcription and replication. Some of the common T>C and C>T transitions in the hypervariable D-Loop and coding regions are potential methylation sites. The D-loop region heteroplasmic variant T16519C that we previously reported as associated with SZ is a possible candidate for methylation for instance. A few studies have investigated mitochondrial neuroepigenetics [39, 40], and mtDNA epigenetic changes have recently been observed in mammalian brains with age and region specific patterns [41]. Thus, rare and common mitochondria sequence variants while not sufficient to cause a classical mitochondrial disease, may be associated with a cascade involving altered energy output in brain depending on the functional variants and loss or gain of methylation sites in the mtDNA especially in the control region (D-Loop).

We paid particular attention to validation of the observed results and confirmed a subset of variants by Sanger sequencing, allelic specific PCR, and allelic specific PCR using LNA primers. We selected 6 heteroplasmic variants with high levels of heteroplasmy for confirmation by Sanger sequencing, while allele specific methods were needed for levels of heteroplasmy between 10 and 20%. A recent pyrosequencing study of mtDNA in 40 Hapmap reference samples reported high levels of heteroplasmy but low confirmation ratios using only Sanger sequencing, even for high heteroplasmy loci (>40%), raising questions about the efficiency of Sanger to detect heteroplasmy [42]. However, the coverage in that study was lower than ours which might explain why some of the heteroplasmic variants observed were false positives. Additionally, misinterpretation of the chromatograms could also explain some of the observed discordant results, like the G1333A locus that is clearly heteroplasmic but was interpreted as homoplasmic [42].

**Homoplasmic variants**

We observed 49 non-synonymous variants at 37 loci that were specific to cases, not found in controls (Table 1), 8 of these were predicted using Polyphen as being possibly/probably
damaging mutations and could potentially have a functional role in mitochondrial dysfunction and psychiatric disorders. In SZ we observed a particularly high number of non-synonymous mutations per subject (22 variants) (Table 2) when compared to non-synonymous variants specific to controls (11 variants). When translated into a Z-score there was an excess of non-synonymous mutations (p = 0.024, two tailed z-score test) in SZ compared to controls. This suggests a higher burden of non-synonymous mutations in SZ and we are conducting new experiments in a larger sample for robust replication. Most of these 49 known non-synonymous variants were not previously associated with any known mitochondrial disorder, ruling out the likelihood that a formal mitochondrial disorder is underlying these psychiatric disorders in this study. However the rate of rare-novel mutations is 5 in 42 psychiatric cases and 2 in 22 controls (Table 3), indicating that our sample surpasses the percentages recently reported for mutation rates in screening mitochondrial genomes from symptomatic patients (3%-6%)[43]. We excluded the common haplogroup defining non-synonymous mutations from our calculations, thus we are cautiously optimistic about excess non-synonymous SNPs dispersed across the mtDNA genome and the trend towards an excess in psychiatric disorders particularly in schizophrenia. In the present mtDNA data, the lack of haplogroup specificity for these mutations supports prior literature that has mainly failed to consistently demonstrate differences between major haplogroups in terms of prevalence of psychiatric disorders [4, 5].

Heteroplasmic Variants

We found novel heteroplasmic loci using NGS. Due to higher sequencing depth and hence higher sensitivity, heteroplasmy as well as somatic mutations are more likely to be detected and reported with NGS as opposed to Sanger sequencing experiments, SNaPshot, Surveyor, etc. [19, 22, 44]. Other technologies such as Sanger sequencing or allelic specific PCR using LNA-primers, must be used to validate heteroplasmy, as we and others find multiple instances of false positives [19, 45].

In general, heteroplasmy can occur in germ-line and become equally distributed throughout many tissues, but it has also been suggested to be a consequence to the effects of reactive oxygen species and other oxidative stress mechanisms inducing substitutions that are not repaired during mtDNA replication [46] or during fission/fusion between mitochondria organelles. On the other hand, recent evidence suggests that somatic mutagenesis is actually influenced by germine mutations that get disseminated by clonal expansion in somatic tissues which can explain also the variable levels of heteroplasmy across the brain and in blood observed in the present study. The present results show equal heteroplasmy in germ-line and brain at some loci, but other loci showed an increase in heteroplasmy in brain with no heteroplasmy found in blood. We report low levels of heteroplasmy in brain tissue not present in blood for three control subjects, underlining the interest in surveying somatic mtDNA variation in brain to uncover mutations possibly involved in neuropsychiatric disorders. Heteroplasmy levels observed in brain exclusively were relatively low usually less than 10% (S8 Table), while a perfect concordance of homoplasmic variants between the two tissues was observed.

We confirmed heteroplasmy at T16519C, a locus previously reported as being hypermutable in multiple haplogroups and that we previously found is associated with SZ [9]. Another locus, T16086C, also showed highly variable levels of heteroplasmy (6.3 to 32.5%) between the brain regions from the same control individual (Fig 2), suggesting that some brain regions might reach detrimental levels of heteroplasmy.

Many diseases can be caused by heteroplasmic mtDNA mutations with clinical manifestation appearing after a certain threshold of mutant heteroplasmy, a concept called phenotypic threshold effect [47]. Studies have shown heteroplasmy within families and between tissues
[48], as well as between cancer and non-cancer tissue from the same individual [22]. Recently, it was shown that heteroplasmy in brain of mice can result in altered metabolic function, as well as altered behavior and cognitive performance [49]. In this study we observed variable levels of heteroplasmy levels between tissues (blood-brain) and within tissue between brain regions from the same individual, pointing to somatic or postzygotic mutations within cells in certain parts of the brain of control subjects. Although no psychiatric cases were assayed for heteroplasmy across brain regions in the present study, we found within controls that heteroplastic mutations can vary between brain regions from the same individual (Fig 2). Low frequency somatic mutations have also been discovered in patients with neurological disorders by whole exome NGS [44]. The authors of the study point to low frequency of mutations in blood as evidence of mosaicism only detectable by high coverage NGS sequencing (>1000X), however as the authors point out they did not have access to brain tissue to determine the distribution of somatic variants associated with the observed neurological alterations [44]. Thus, we will sequence additional brain samples from subjects with psychiatric disorders, to address heteroplasmy across brain regions as a potential indicator of mitochondrial dysfunction.

Multiplasmy

Multiplasmy was observed in three loci located in the D-loop region (D310, D16189 and 514–523(CA)n). Two of these multiplasmy loci consist of poly cytosine tracts interrupted by a thymidine at positions 310 and 16189. These two regions are known to be highly variable and are potentially associated with mitochondria copy number and neurological disorders [29, 50, 51]. Multiplasmy at these loci has been confirmed by cloning and Sanger sequencing [52, 53]. Multiplasmy was also observed at the 514–523 (CA)n locus (S2 Fig), a phenomenon previously reported and confirmed by Sanger sequencing [54]. This multiplasmy occurs 1.5 times more frequently in psychiatric cases compared to controls at 514 CA dinucleotide repeat, albeit in a small sample this is not significant (p = 0.14). Multiplasmy in this region can perhaps induce differences in transcription, since this region is at the border of binding of TFAM1 to mtDNA at bp 523–550. We observed variation in multiplasmy levels versus wild type at this locus across brain regions (S2 Fig), but multiplasmy was present in all the brain regions examined. The increased multiplasmy in psychiatric brains, while not significant, is perhaps another indicator suggesting an involvement of genetic instability of mtDNA in the predisposition to psychiatric disorders.

In conclusion, we observed several new and rare mitochondrial non-synonymous mutations in psychiatric cases and excess non-synonymous mtDNA mutations in persons with schizophrenia (Table 1). These findings support the hypothesis that common and rare mitochondrial mutations can play a role in psychiatric disorders, especially schizophrenia [9, 14–16]. Additionally, in a preliminary analysis, we observed a higher proportion of multiplasmic and heteroplasmic burden primarily in the hyper-variable region in psychiatric cases which was not significantly different from controls. Although speculative, these data point to a higher genomic instability in the mtDNA of psychiatric patients. It is widely known that mtDNA has a higher mutation rate than nuclear DNA, lacks protective histones, and undergoes genomic replication independent of cell division, all of which contribute to functional mutations. Our observations are in agreement with recent reports of an excess of non-synonymous variants in nuclear genes [55] and a higher rate of de novo mutations in nuclear genes in schizophrenia [56], as well as somatic mutations present in neurological disorders [44, 57, 58]. A larger study is required in multiple brain regions and blood to directly test our hypothesis that rare and novel mutations in mtDNA are enriched in psychiatric cases.
Materials and Methods

Subjects

Anonymized and de-identified post-mortem human brain samples were obtained from the University of California, Irvine Brain Bank (UCIBB; www.vawterlab.com). This study was approved by the Institutional Review Board (IRB) of the University of California, Irvine. Signed informed consent was obtained from next of kin. A total of 14 BD (5F/9M, 54±12 yr), 20 controls (5F/15M, 50±18 yr), 15 MDD (8F/7M, 45±12 yr), 14 SZ (7F/7M, 44±9), as well as 6 methamphetamine users with no axis I diagnosis (METH; 4F/2M, 42±8 yr), were analyzed (S1 and S2 Tables) resulting in 121 complete mtDNA sequences from 69 subjects. We presented homoplasmic NGS data for 23 of the subjects (cohort 1) in a recent paper [9], in this study we explored also heteroplasmic and multiplasmic mutations in 46 new subjects (cohort 2). In summary, all 69 subjects had the DLPFC sequenced and for a subset of subjects blood as well as other brain regions depending on availability of tissue were also sequenced.

Eleven brain regions were dissected on dry ice from the left hemisphere according to visible landmarks near the regions of interest. DNA was extracted from the following brain regions: anterior cingulate cortex (ACC), amygdala (AMY), caudate nucleus (CAUN), cerebellum (CB), dorsolateral prefrontal cortex (DLPFC), hippocampus (HIPP), nucleus accumbens (NACC), orbitofrontal cortex (OFC), putamen (PUT), substantia nigra (SN), and thalamus (THAL) for 11 subjects and whole blood was also obtained for three of those subjects. DNA was extracted from 25 mg of dissected brain tissue using the DNeasy Blood and Tissue Kit (QIAGEN), according to the manufacturer’s protocol. Previously, DNA was extracted from DLPFC samples (cohort 1) using the phenol phase of a Trizol protocol and precipitated with ethanol [59]. Additional details are available in S1 Methods.

Next-Generation Sequencing Analysis

Mitochondrial NGS was performed as described previously [9]. Briefly, two overlapping mtDNA fragments were PCR amplified, purified, and sequenced using standard manufacturer’s protocols. Reads from Illumina GAII (cohort 1) and HiSeq (cohort 2) were aligned to the mitochondrial revised Cambridge Reference Sequence (rCRS; GenBank accession number NC_012920)[27]. We defined homoplasmic mutations as genetic variants for which the major allele was different from the rCRS and constituted 95% to 100% of the reads. Heteroplasmic mutations were therefore defined as any variant for which the major allele was <95% of the total of the observed alleles. For both homoplasy and heteroplasy we filtered out variants with coverage lower than 200 reads. These cutoffs were implemented based on our experience with false positive mutations in problematic regions of the mtDNA. All the mutations reported were successfully confirmed by Sanger sequencing. We focused on results from the DLPFC.
and SN for reasons of clarity and space (n = 69 unique subjects). We determined whether the
mutations were novel or rare by investigating their presence in curated online databases such
as MITOMAP containing more than 26,850 Genbank mtDNA sequences [60], mtDB [61], and
PhyloTree [28], and also by searching individual genealogical studies reporting mtDNA muta-
tions using Google as suggested [62]. We also compared our sequence variant results to refer-
dence databases, 1000 Genomes mitochondrial variants, and to the Phylotree database that
contains over 16,500 mtDNA genomes which overlaps MITOMAP. The effect of amino acid
substitutions was determined using Polyphen [63, 64].

Quality control was critical especially for heteroplasmic variants. Heteroplasmic A>C vari-
ants were observed in many brain samples in the same loci of the mtDNA genome. However,
we were unable to confirm these variants using Sanger sequencing or allele specific PCR with
locked nucleic acids (Table 5). Some of these error hotspots have been previously reported
using the Illumina platform [19]. We noted a disproportionate number of A>C heteroplasmic
substitutions (148 out of 401 heteroplasmic variants) which was more than what would be ex-
pected, as transversions occur less frequently than transitions. Two previously reported error
hotspots in Illumina NGS [19], the A3492C and A10306C loci, and a transversion never re-
ported at position A6419C were frequently observed (Table 5). Three other non A/C hetero-
plasmic positions that did not validate were T3488A, T6415A, and G9801T. Closer
examination of the sequence surrounding the A>C sequencing error hotspots revealed a com-
mon motif of at least two A nucleotides followed by at least two C nucleotides (Table 5). We
choose for validation 6 of the heteroplasmic variants with heteroplasmy levels of more than
10% to be confirmed by Sanger sequencing and they were all successfully confirmed.

Supporting Information

S1 Fig. Phylogenetic tree of mtDNA variants observed in the present cohort. The first letter
of the subject label corresponds to the diagnosis (B: bipolar disorder; S: schizophrenia; M:
major depression; D: drugs (methamphetamine)), the number is the age of the subject, and the
letter after the age is the gender (F: female; M: male).

S2 Fig. The ratio of the deletion to wild type at 514 CA across brain regions. Note for both
subjects that the ratio is quite variable from 1.35–3.24, while the other subject is 1.57–2.73.

S1 Methods.

S1 Table. Subject demographics by NGS platform.

S2 Table. Number of samples processed in brain and blood for next generation sequencing.

S3 Table. Distribution of the 984 homoplasmic SNPs (compared to the rCRS) observed in
65 DLPFC samples sequenced (two DLPFC were from METH subjects).

S4 Table. Total number of non-synonymous mtDNA homoplasmic sequence substitutions
in 65 DLPFC samples.
S5 Table. The distribution of subjects by number of non-synonymous coding mutations categorized by diagnosis. There was a non-significant excess of 5+ mutations in cases compared with controls (Fishers Exact Test, one-sided p = 0.068).

S6 Table. Homoplasmic mutations in 12S and 16S rRNA genes. In gray are the variants only present in cases but not in controls.

S7 Table. Heteroplasmic variants in the DLPFC were mainly clustered in the D-Loop region.

S8 Table. Heteroplasmic variants that were present at various levels in brain tissue but undetectable in blood.

Author Contributions
Conceived and designed the experiments: AS BR CM MVO PB RMM JDB AFS SJW HA WEB MPV. Performed the experiments: AS BR CM MVO MPV. Analyzed the data: AS BR CM MVO PB MPV. Wrote the paper: AS BR CM MVO PB RMM JDB AFS SJW HA WEB MPV.

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