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RESEARCH ARTICLE

Downregulation of the Expression of Mitochondrial Electron Transport Complex Genes in Autism Brains

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Keywords
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Abstract

Mitochondrial dysfunction (MtD) and abnormal brain bioenergetics have been implicated in autism, suggesting possible candidate genes in the electron transport chain (ETC). We compared the expression of 84 ETC genes in the post-mortem brains of autism patients and controls. Brain tissues from the anterior cingulate gyrus, motor cortex, and thalamus of autism patients (n = 8) and controls (n = 10) were obtained from Autism Tissue Program, USA. Quantitative real-time PCR arrays were used to quantify gene expression. We observed reduced expression of several ETC genes in autism brains compared to controls. Eleven genes of Complex I, five genes each of Complex III and Complex IV, and seven genes of Complex V showed brain region-specific reduced expression in autism. *ATP5A1* (Complex V), *ATP5G3* (Complex V) and *NDUFA5* (Complex I) showed consistently reduced expression in all the brain regions of autism patients. Upon silencing *ATP5A1*, the expression of mitogen-activated protein kinase 13 (*MAPK13*), a p38 MAPK responsive to stress stimuli, was upregulated in HEK 293 cells. This could have been induced by oxidative stress due to impaired ATP synthesis. We report new candidate genes involved in abnormal brain bioenergetics in autism, supporting the hypothesis that mitochondria, critical for neurodevelopment, may play a role in autism.

INTRODUCTION

Autism is a complex pervasive developmental disorder characterized by deficiencies in social interaction and communication, and repetitive and stereotyped behaviors. It is heterogeneous and belongs to a group of neurodevelopmental disorders, collectively known as autism spectrum disorders (ASDs) that also include Asperger syndrome and pervasive developmental disorder-not otherwise specified (PDD-NOS). The abnormalities are usually identified in the early years of childhood. According to a recent report, the prevalence of ASD has risen to 1 in 110, with a male to female ratio of 4.5:1 (51).

A plethora of evidence from biochemical (3), anatomical (30) and neuroimaging (26, 39) studies has implicated a disturbed brain bioenergetic metabolism in the pathogenesis of autism. Abnormal levels of biomarkers indicative of energy metabolism have been observed in autistic patients (3, 6, 26, 41). Magnetic resonance spectroscopy studies have shown in the brain of autism patients altered levels of metabolites relating to brain bioenergetics (14, 39).

Most of the adenosine triphosphate (ATP), the source of chemical energy in cells, is generated by mitochondria, which serve as the energy powerhouses of eukaryotic cells. Abnormal brain bioenergetics, therefore, indicates an involvement of mitochondrial dysfunction (MtD) in the pathogenesis of ASD (33). Furthermore, diminished levels of ATP have been observed in autistic brains (39).

In a systematic review and meta-analysis, Rossignol and Frye (54) reported MtD in 5.0% of children with ASD. Comorbid features such as developmental regression, learning disabilities, mental retardation, seizures, motor delay, neurological problems and gastrointestinal abnormalities have been found to be significantly more prevalent in children with ASD/MtD as compared with the general ASD population (12, 53, 54). In addition, defective lymphocytic mitochondria have been observed in autistic children (26). Several studies have suggested that antioxidants (eg, coenzyme Q10) and/or nutritional supplements (eg, carnitine, vitamin B) could be beneficial in the treatment of a fraction of children with ASD/MtD (21, 63). Rats induced for MtD have been found to exhibit certain brain, behavioral and metabolic changes characteristic of ASD, such as microglial activation, reduced levels of glutathione, repetitive behaviors, social interaction deficits, hyperactivity and oxidative stress (OS) (34, 35, 57).

Mitochondrial energy production, which occurs through a process called oxidative phosphorylation, requires the action of various respiratory enzyme complexes termed as the electron transport chain (ETC) located in the inner mitochondrial membrane. There are five ETC complexes: (i) Complex I (NADH dehydrogenase); (ii) Complex II (succinate dehydrogenase), (iii) Complex III (cytochrome *bc1* complex), (iv) Complex IV [cytochrome *c* oxidase (COX)]; and (v) Complex V (ATP synthase). In a recent study, Chauhan *et al* (12) observed brain region-specific deficits in ETC complexes in autistic children. They had examined the protein expression of each ETC complex, but not the expression of individual proteins in each complex.

In the present study, we aimed at analyzing the expression of genes involved in each ETC complex. Using the post-mortem brains of autistic patients and healthy controls, we compared the expression of 84 genes belonging to the five ETC complexes. The differential expression of *ATP5A1*, one of the downregulated genes in autism, was verified at the protein level. Furthermore, we silenced *ATP5A1* and studied its effect on the expression of its interacting proteins in HEK 293 cells.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Hamamatsu University School of Medicine.

Gene expression studies of human post-mortem brains

Post-mortem brain tissues

Post-mortem brain samples of autism patients and healthy controls were provided by Autism Tissue Program (Princeton, NJ, USA; http://www.autismtissueprogram.org), NICHD Brain and Tissue Bank for Developmental Disorders (NICHD BTB; Baltimore, MD, USA; http://medschool.umaryland.edu/btbank/), and Harvard Brain Tissue Resource Center (HBTRC; Belmont, MA, USA; http://www.brainbank.mclean.org/). Frozen tissue samples from the following brain regions were used in the study: (i) anterior cingulate gyrus (ACG); (ii) motor cortex (MC); and (iii) thalamus (THL). Demographic characteristics of the samples (ACG: 8 autism, 9 controls; MC: 7 autism, 8 controls; THL: 8 autism, 8 controls) are described in Table 1.

The differences in age and post-mortem interval (PMI) between the autism and control groups were examined by *t*-test. Fisher's exact test was used to examine the differences in sex distribution between the two groups (Table 2).

RNA extraction

The brain tissues were homogenized by ultrasonication, and total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocol. The RNA samples were further purified using RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 and 280 nm) of RNA were estimated with a NanoDrop ND-1000 Spectrophotometer (Scrum, Tokyo, Japan). As per the requirements for the subsequent array experiment, the following criteria were met for all of the RNA samples: (i) A260:A230 ratio, >1.7; (ii) A260:A280 ratio, between 1.8 and 2.0; and (iii) concentration of total RNA, >40 ng/μL.

First-strand cDNA synthesis

First-strand cDNA was synthesized from 500 ng of total RNA using the RT^2 First Strand Kit (SABiosciences, Frederick, MD,

Table 1. Post-mortem brain tissue information. Abbreviations: PMI = post-mortem interval; NA = not available; M = male; F = female; ACG = anterior cingulate gyrus; MC = motor cortex; THL = thalamus.

Sample ID*	Diagnosis	Age (years)	Gender	PMI (hours)	Race	Cause of death	Brain regions†
1065	Control	15	M	12	Caucasian	Multiple injuries	ACG, THL
1297	Control	15	M	16	African American	Multiple injuries	ACG, MC, THL
1407	Control	9	F	20	African American	Asthma	ACG, MC, THL
1541	Control	20	F	19	Caucasian	Head injuries	ACG, MC, THL
1649	Control	20	M	22	Hispanic	Multiple injuries	ACG, MC, THL
1708	Control	8	F	20	African American	Asphyxia, multiple injuries	ACG, MC, THL
1790	Control	13	M	18	Caucasian	Multiple injuries	ACG
1793	Control	11	M	19	African American	Drowning	ACG, MC, THL
1860	Control	8	M	5	Caucasian	Cardiac arrhythmia	ACG
4543	Control	28	M	13	Caucasian	Multiple injuries	MC, THL
4722	Control	14	M	16	Caucasian	Multiple injuries	MC
797	Autism	9	M	13	Caucasian	Drowning	ACG, THL
1638	Autism	20	F	50	Caucasian	Seizure	ACG, MC, THL
4231	Autism	8	M	12	African American	Drowning	ACG, MC, THL
4721	Autism	8	M	16	African American	Drowning	ACG, MC, THL
4899	Autism	14	M	9	Caucasian	Drowning	ACG, MC, THL
5000	Autism	27	M	8.3	NA	NA	ACG, MC, THL
6294	Autism	16	M	NA	NA	NA	ACG, MC, THL
6640	Autism	29	F	17.83	NA	NA	ACG, MC, THL

*Autism Tissue Program identifier.

†Brain regions for which each sample was available.

Table 2. Demographic characteristics of post-mortem brain samples. Abbreviations: SD = standard deviation; ACG = anterior cingulate gyrus; MC = motor cortex; THL = thalamus; M = male; F = female.

Brain region	Age (mean ± SD)			Post-mortem interval (mean ± SD)			Gender		
	Control	Autism	*P*-value*	Control	Autism	*P*-value*	Control	Autism	*P*-value†
ACG	13.22 ± 4.68	16.38 ± 8.33	0.344	16.78 ± 5.26	18.02 ± 14.52	0.815	6M, 3F	6M, 2F	1.000
MC	15.63 ± 6.74	17.43 ± 8.40	0.652	18.13 ± 2.90	18.86 ± 15.71	0.899	5M, 3F	5M, 2F	1.000
THL	15.75 ± 6.71	16.38 ± 8.33	0.871	17.63 ± 3.58	18.02 ± 14.52	0.942	5M, 3F	6M, 2F	1.000

**t*-test.

†Fisher's exact test (two sided).

USA), following the manufacturer's protocol. The kit contains an effective genomic DNA elimination step and a built-in external RNA control that helps monitor reverse transcription efficiency, and tests for contaminating inhibitors during the quantitative PCR (qPCR).

Real-time qPCR

The Human Mitochondrial Energy Metabolism RT^2 Profiler^{TM} PCR Array (SABiosciences) was used for quantifying the expression of 84 ETC genes in post-mortem brain samples. In addition to the 84 genes, the array has five reference genes [beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin beta (ACTB)], three reverse transcription controls (RTCs), three positive PCR controls (PPCs) and one genomic DNA control (GDC) making up to a total number of 96 assays. The details of the genes in the array are provided in Supporting Information Tables S1 and S2. The 384-well format array includes four replicates of each of the 96 assays. The array makes use of SYBR Green method of quantitative real-time PCR analysis. PCR reactions were carried out according to the manufacturer's protocol in ABI PRISM 7900HT SDS [Applied Biosystems (ABI), Foster City, CA, USA].

Data analysis

The threshold cycle (Ct) values obtained from qPCR were analyzed by the ΔΔCt method using the Microsoft Excel-based program RT^2 Profiler PCR Array Data Analysis (SABiosciences). It calculates: (i) ΔCt of each gene = Ct of gene of interest (GOI)—average Ct of chosen reference genes; (ii) ΔΔCt for each gene across two groups; ΔΔCt = ΔCt autism group—ΔCt control group; and (iii) fold change for each gene from control group to autism group as $2 \wedge (-\Delta\Delta Ct)$. Based on Kolmogorov–Smirnov test, the expression of all genes was found to follow a normal distribution. Therefore, *t*-test, which was also the default option in our data analysis program, was used to examine the significance of difference in gene expression between control and autism groups.

The statistical program performs the following functions also: (i) interprets all Ct values ≥ 35 as a negative call; (ii) examines genomic DNA contamination in the samples based on the Ct of GDC; Ct <35 indicates genomic DNA contamination; (iii) examines the presence of impurities in RNA samples based on the Ct value of PPC; Ct should be 20 ± 2 on each array, and should not vary by more than two cycles between the arrays being compared; and (iv) interprets any inhibition of reverse transcription based on the Ct values of RTC and PPC; ΔCt (Ct RTC-Ct PPC) < 5 indicates that there is no apparent inhibition.

Western blot analysis

By using Western blot analysis, we examined any differential expression of ATP5A1 protein in the ACG of autism patients (n = 8) compared to controls (n = 9). The brain samples were homogenized in RIPA Buffer (Sigma-Aldrich, Tokyo, Japan). The total protein in the lysate was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). A total of 10 μg of each sample was separated on 7.5% sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE). The separated proteins were electroblotted onto a PVDF membrane (Millipore, Billerica, MA, USA), blocked and incubated with the primary antibody at 4°C overnight. The primary antibodies used were: (i) anti-ATP5A (ab14748, abcam, Tokyo, Japan) at 1:1000 dilution for the detection of ATP5A1; and (ii) anti-GAPDH (ab9484, abcam) at 1:5000 dilution for the detection of GAPDH, which was used as the loading control. Following primary antibody incubation, the blots were washed and incubated with 1:15 000 diluted IRDye 700 DX conjugated secondary antibody (610-730-124, Rockland, Gilbertsville, PA, USA). The blots were scanned in Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Using the Grid tool of Odyssey Analysis software v2.1 (LI-COR), the trimmed mean signal intensities of protein bands were quantified. The signal intensity of ATP5A1 was normalized against that of GAPDH. The difference in protein expression between the control and autism groups was estimated by *t*-test.

Suitable antibodies against ATP5G3 and NDUFA5 were unavailable.

Protein-interacting partners of ATP5A1

Proteins interacting with ATP5A1 were identified from the data available in Human Protein Reference Database (HPRD; http://www.hprd.org/), BioGRID (http://thebiogrid.org/) and IntAct (http://www.ebi.ac.uk/intact/). From among the several proteins listed, four were selected as they were predicted as interacting proteins of ATP5A1 by at least two of the aforementioned programs. These include p38 mitogen-activated protein kinase 13 (MAPK13), 14-3-3 beta (YWHAB), 14-3-3 gamma (YWHAG) and 14-3-3 zeta (YWHAZ). In addition, three other p38

mitogen-activated protein kinases, MAPK11, MAPK12 and MAPK14, were also included in the study.

Silencing of *ATP5A1* by RNA interference (RNAi)

Cell culture

HEK 293 cells were grown at 37°C, with 5% CO2 in Dulbecco's modified Eagle's medium—low glucose (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Silencing of ATP5A1 by RNAi

HEK 293 cells, at 30% confluency, were transfected with s1767, an *ATP5A1*-specific small interfering RNA (siRNA; *Silencer* Select Predesigned and Validated siRNAs, ABI), using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. Transfection with 200 pmol of s1767 resulted in 86% silencing of *ATP5A1* with minimal cytotoxicity, when grown for 48 h.

The effect of *ATP5A1* silencing on the gene expression of its interacting proteins (*MAPK11*, *MAPK12*, *MAPK13*, *MAPK14*, *YWHAB*, *YWHAG* and *YWHAZ*) was determined by comparing their gene expression between s1767-transfected cells (five replicates) and negative control siRNA (Silencer Select Negative Control #1 siRNA, ABI)-transfected cells (five replicates) by qPCR method.

RNA extraction was carried out in accordance with the protocol in Section 2.1.2. First-strand cDNA was synthesized from total RNA with oligo(dT) primer, using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA), following the manufacturer's protocol. Real-time qPCR analysis was performed by TaqMan method in ABI PRISM 7900 Sequence Detection System (ABI). TaqMan primer/probes for the GOI (*ATP5A1*: Hs00900735_m1; *MAPK13*: Hs00559623_m1; *YWHAB*: Hs00793604_m1; *YWHAG*: Hs00705917_s1; *YWHAZ*: Hs03044281_g1) and for the reference gene, *GAPDH* (Predeveloped TaqMan Assay Reagent) were purchased from ABI. All reactions were performed in duplicate, according to the manufacturer's protocol. C_T, which reflects the mRNA expression levels, was determined, and the C_T of the GOI of each sample was normalized to the corresponding C_T for *GAPDH*, by calculating ΔC_T (ΔC_T = GOI C_T – *GAPDH* C_T), to obtain the relative mRNA expression of the GOI. Quantification of the expression of the GOI was calculated as $\Delta\Delta C_T$ ($\Delta\Delta C_T = \Delta C_T$ ATP5A1 RNAi – ΔC_T negative control RNAi). The fold change in gene expression between the two groups was determined by calculating $2^{-\Delta\Delta C_T}$. Any significant difference in the expression of the GOI between autism and control groups was assessed by *t*-test.

RESULTS

Gene expression studies of human post-mortem brains

In the qPCR experiment, the Ct of GDC was >35 for all the samples, indicating that genomic DNA contamination, if any, was too low to affect the gene expression results. The Ct of PPC was 20 ± 2 for all the arrays, showing the apparent absence of impurities in the RNA samples. There was also no indication of any inhibition of reverse transcription reaction, as ΔCt (Ct RTC-Ct PPC) was <5 for all the samples. Reference genes for normalization of gene expression were chosen separately for each of the brain regions, such that the selected genes did not show any significant difference in expression between the control and autism groups. The reference genes selected for the various brain regions were: (i) *RPL13A*, *GAPDH* and *ACTB* for ACG; (ii) *B2M*, *RPL13A*, *GAPDH* and *ACTB* for MC; and (iii) *B2M*, *RPL13A*, *GAPDH* and *ACTB* for THL.

There was no significant difference in age, PMI or sex distribution between the control and autism groups in any of the brain regions (Table 2). We observed reduced expression of several ETC genes in the autism group compared to the control group (Table 3). Eleven genes belonging to Complex I, five genes each belonging to Complex III and Complex IV, and seven genes belonging to Complex V showed reduced expression in the various brain regions of autism patients. There was no significant alteration in the expression of Complex II genes between the control and autism groups. Overall, the expression of 11 ETC genes in ACG, 12 genes in MC and 19 genes in THL was downregulated in autism patients. However, none of the *P*-values withstood multimarker testing (conventional Bonferroni approach). The expression of *ATP5A1* (Complex V), *ATP5G3* (Complex V) and *NDUFA5* (Complex I) was reduced in all the three brain regions of autism patients.

Western blot analysis

We compared the protein expression of ATP5A1 in the ACG of autism patients and control subjects. In concordance with mRNA levels, ATP5A1 expression was found to be reduced in autism patients compared to the controls ($P = 0.041$) (Figure 1). The expression of ATP5A1 in MC and THL could not be examined due to the unavailability of sufficient tissue samples.

Effect of *ATP5A1* silencing on the gene expression of its interacting proteins

The gene expression of ATP5A1-interacting proteins (*MAPK11*, *MAPK12*, *MAPK13*, *MAPK14*, *YWHAB*, *YWHAG* and *YWHAZ*) was compared in *ATP5A1*-silenced HEK 293 cells and control cells. The expression of *MAPK13*, a stress-activated p38 MAPK, was found to be significantly higher in the *ATP5A1*-silenced cells compared to the negative control ($P = 0.018$) (Figure 2). There was no significant difference in the expression of other proteins between A*TP5A1*-silenced cells and control cells.

DISCUSSION

We report brain-region specific decrease in the expression of mitochondrial ETC genes in autism. This is the first comprehensive study evaluating the expression of individual ETC genes in autism brain. Previously, defects of ETC complexes had been reported in the muscle biopsies (49), buccal swabs (21) and lymphocytes (26) of autism patients. Recently, Chauhan *et al* (12) observed reduced levels of Complexes III and V in the cerebellum, of Complex I in

Table 3. Genes with altered expressions in autism post-mortem brains.

Gene	Anterior cingulate gyrus		Motor cortex		Thalamus	
	Fold difference	*P*-value*	Fold difference	*P*-value*	Fold difference	*P*-value*
*ATP5A1***	–1.753	0.034	–1.762	0.045	–1.857	0.031
*ATP5B***					–1.939	0.038
*ATP5F1***			–2.326	0.022	–1.672	0.036
*ATP5G3***	–2.378	0.022	–2.941	0.015	–2.637	0.007
*ATP5H***	–4.476	0.044				
*ATP5J***					–1.742	0.016
*ATP6V0A2***			–1.733	0.009		
COX5A¶	–1.664	0.043				
COX6A1¶	–1.677	0.018	–1.469	0.043		
COX7A2¶					–1.662	0.042
COX7B¶					–1.521	0.036
COX8A¶	–1.467	0.049				
CYC1§					–1.586	0.045
NDUFA2†			–1.635	0.009		
NDUFA4†	–1.769	0.016			–1.574	0.049
NDUFA5†	–1.800	0.037	–2.168	0.005	–1.885	0.028
NDUFA8†	–1.670	0.043	–2.197	0.007		
NDUFAB1†	–1.599	0.041			–1.572	0.038
NDUFB3†					–1.823	0.017
NDUFB4†			–1.710	0.018		
NDUFB5†			–2.133	0.017	–1.792	0.015
NDUFB6†					–1.996	0.022
NDUFS2†					–1.554	0.043
NDUFS4†					–1.466	0.026
UQCRC1§			–2.128	0.014		
UQCRC2§					–1.490	0.045
UQCRFS1§			–1.819	0.049	–1.706	0.030
UQCRH§	–1.621	0.047			–1.902	0.034

**t*-test.
†Complex I (NADH-coenzyme Q reductase).
§Complex III (coenzyme Q-cytochrome c reductase).
¶Complex IV (cytochrome c oxidase).
**Complex V (ATP synthase).

the frontal cortex, and of Complexes II and III in the temporal cortex of autistic children. In concordance with this, we observed brain region-specific downregulation of ETC genes belonging to Complexes I, III, IV and V in autism.

The expression of ETC genes was analyzed in the ACG, MC and THL. The ACG has been found to be involved in emotion formation and processing, learning and memory (9, 61), the MC in planning, control and execution of voluntary motor functions (20),

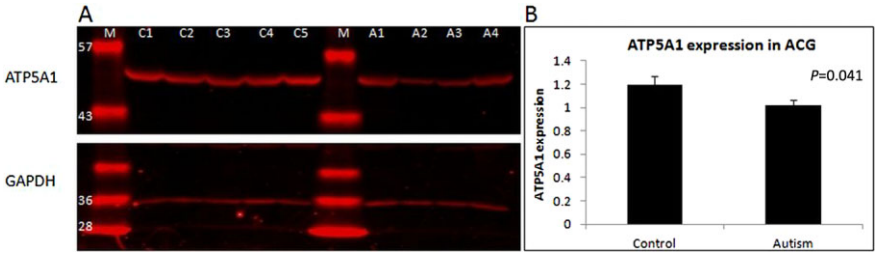

Figure 1. *Comparison of the protein expression of ATP5A1 in the anterior cingulate gyrus (ACG) of autism patients and healthy controls.* **A.** Representative gel showing the protein levels of ATP5A1 (55 kDa) and of the loading control GAPDH (36 kDa) in control and autism brains. C1–C5: control samples; A1–A4: autism samples; M: molecular weight marker (molecular weight in kDa is given along the left side of each band). **B.** ATP5A1 expression was significantly reduced in autism brains compared to controls ($P = 0.041$). *y*-axis of the graph represents the relative expression of ATP5A1 normalized to GAPDH.

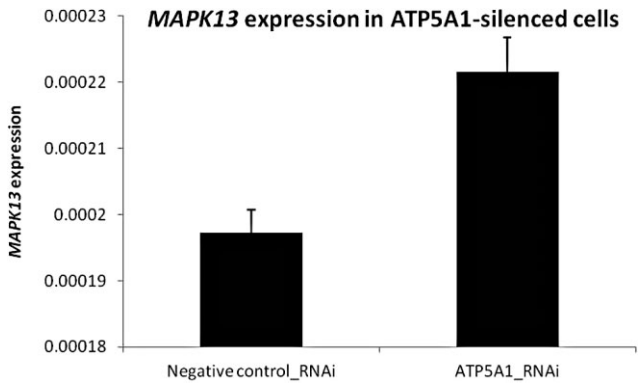

Figure 2. Comparison of *MAPK13* expression in ATP5A1-silenced and negative control siRNA-transfected HEK 293 cells. The expression of *MAPK13* was found to be significantly higher in the *ATP5A1*-silenced cells compared to the negative controls ($P = 0.018$). *y*-axis of the graph represents the relative expression of *MAPK13* normalized to *GAPDH*.

and the THL in the processing and relaying of sensory information (38). Abnormalities of the anterior cingulate have been implicated in impairments of cognitive control (2), social orientation (43), social target detection (17) and response monitoring (13, 62) in autism. Defects of the MC, such as increased white matter volume, have been observed in autism (42, 58). Reduced thalamic volume has been reported in autistic individuals (19, 64), and the impairments of auditory, tactile and visual stimuli processing have been attributed to THL abnormalities (40).

In the ACG, MC and THL, the expression of *NDUFA5* (Complex I), *ATP5A1* (Complex V) and *ATP5G3* (Complex V) was consistently reduced in autism patients. These genes are vital constituents of the ETC. NDUFA5, a component of NADH dehydrogenase (Complex I), is involved in building up the electrochemical potential required to produce ATP. A recently report shows suggestive evidence for the association of *NDUFA5* with autism (37). *ATP5A1* and *ATP5G3* are components of ATP synthase, which produces energy via ATP synthesis.

We observed, in addition to *NDUFA5*, reduced expression of several Complex I genes in autism. Reduction of Complex I has been found to increase OS by contributing to the production of free radicals (5, 28). Chauhan *et al* (12) observed downregulation of Complex II (succinate dehydrogenase) in the temporal cortex of autistic children. While we did not examine gene expression in the temporal cortex, we could not find altered expression of Complex II genes in the ACG, MC or THL of autism patients. Several genes in cytochrome *bc1* complex, the third complex in ETC, showed reduced expression in autism. Defects of Complex III could be involved in the generation of reactive oxygen species (ROS), leading to neuronal cell death (29). Several genes belonging to Complex IV (COX) were downregulated in autism. Defects in COX can result in severe, often fatal metabolic disorders, which usually manifest in early childhood, predominantly in tissues with high energy demands such as brain (33, 48). We found, in addition to *ATP5A1* and *ATP5G3*, decreased expression of several Complex V (ATP synthase) genes in autism. Low levels of ATP observed in the brain (39) and plasma (1) of autism patients could be attributed to the reduced expression of genes contributing to ATP synthesis.

While in their previous study Chauhan *et al* (12) observed ETC defects in autistic children aged 4–10 years, deficits were not observed in adults (14–39 years). It was suggested that the deficits observed in autistic children might readjust to normal levels in adulthood. However, among the 19 samples in our study, only 6 were of >10 years of age. Our results suggest that ETC deficits might persist into adulthood.

The exact mechanism by which MtD contributes to the etiology of autism is not clear. ATP produced via oxidative phosphorylation in the mitochondria is essential for sustaining electrophysiological activity, cell signaling and structural integrity of brain (7, 18, 52). A minor proportion of oxygen involved in oxidative phosphorylation may be used up for generating ROS (60). Generation of ROS occurs mainly at Complex III (30), with a little contribution from Complex I (5, 28). Defective ETC could increase OS in the brain (12). Increasing evidence suggests a role for OS in the development and clinical manifestation of autism (10, 11, 27, 68). The brain is highly vulnerable to OS due to its limited antioxidant capacity and higher energy requirements (4). As mitochondria are localized in the synapses, MtD could adversely affect synaptic transmission. ETC defects, if occurring during critical developmental stages, could damage the neuronal and glial cells in the brain, leading to neuroinflammation, excitotoxicity, impairments of neural circuit connectivity and plasticity, and/or abnormalities in neurodevelopment (65). This might underlie the cognitive, language and behavioral deficits observed in autism.

OS is known to activate stress signaling pathways, thereby inducing apoptosis in a variety of cell types. Intracellular cell death signaling cascade involving p38 MAPKs is one such pathway activated by OS (36, 44). Upon silencing *ATP5A1* in HEK 293 cells, the expression of *MAPK13*, a p38 MAPK, was found to be increased. MAPK13 is one of the four p38 MAPKs that play an important role in the cascades of cellular responses evoked by extracellular stress stimuli. The upregulated expression of *MAPK13* could have been induced by the OS resulting from downregulated expression of ATP5A1. Further research into the mechanisms of stress and apoptosis pathways stimulated by impaired ATP synthesis is warranted.

It is a matter of debate whether MtD is primary or secondary to autism. Converging evidence suggests an association between ASD and MtD (22, 23, 63), with special emphasis on the impairment of mitochondrial energy metabolism (34). ASD patients have often been found to manifest biochemical or neuropathological traits linked with altered mitochondrial function (3, 6, 14, 15, 24, 31, 39, 41, 46). As MtD often results in central nervous system dysfunction, leading to cognitive and behavioral abnormalities, ASD could be an important clinical presentation of MtD (45). However, the clinical features, and the biochemical and genetic abnormalities in ASD patients with an underlying MtD have been heterogeneous. In addition, several of the biochemical abnormalities occur in the absence of any genetic alterations directly impacting vital mitochondrial functions (47). On the contrary, MtD might also occur as secondary to other pathophysiological processes involved in autism such as immune dysregulation, OS and altered calcium homeostasis (47). Even though it is possible that a greater proportion of individuals with ASD might have MtD at the genetic level, it may not be manifested clinically.

Factors inherent in post-mortem brain studies, and beyond the investigator's control, might have influenced our results. We did not

have sufficient data regarding brain pH. However, large-scale gene analysis showed that brain pH or PMI has no significant correlation with RNA integrity (8, 50). pH could be lower in the post-mortem brains of individuals who suffered prolonged agonal states, such as in respiratory arrest, multi-organ failure and coma (32). However, the cause of death was sudden for most of the subjects included in our study (Table 1). So, we assume that brain pH is unlikely to have affected gene expression. The other concern is the effect of medication, as several drugs such as antidepressants, antipsychotics and selective serotonin reuptake inhibitors are known to inhibit oxidative phosphorylation (16, 55, 66). In this study, information regarding medication (drug doses unknown) was available for only three autism patients, two of whom had received more than two classes of drugs. Therefore, it was difficult to examine the effects of medication on gene expression. Nevertheless, concordant results (not shown) were obtained when these three samples were excluded from the analysis. Another matter of concern is that the cause of death for a majority of the autism patients was drowning, a hypoxic event. Hypoxia is generally known to downregulate the expression and activity of ETC (59); however, it is also reported to rapidly increase mitochondrial biogenesis (25, 67) along with an increased expression of the respiratory enzyme COXIV (67). Delayed susceptibility of brain mitochondria to prolonged hypoxia has also been observed (56). These are considered as an endogenous neuroprotective response of the brain. Therefore, it is unlikely that hypoxia influenced our results significantly. Moreover, reduced expression of ETC complexes has already been reported in autistic children in a study (12) in which the number of deaths due to drowning was similar for autism (n = 3) and control (n = 2) groups. Our results are in accordance with this study.

Because of the availability of potential treatment options for MtD, investigation into the early detection methodologies of MtD could turn out to be useful in autism treatment. If detected in early stages, treatment strategies aimed at reducing its impact may be adopted.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Details of the genes included in Human Mitochondrial Energy Metabolism PCR Array
Table S2. Functional groupings of the 84 electron transport complex genes included in Human Mitochondrial Energy Metabolism PCR Array