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## Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism

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### Abstract

Mitochondria play important roles in generation of free radicals, ATP formation, and in apoptosis. We studied the levels of mitochondrial electron transport chain (ETC) complexes, that is, complexes I, II, III, IV, and V, in brain tissue samples from the cerebellum and the frontal, parietal, occipital, and temporal cortices of subjects with autism and age-matched control subjects. The subjects were divided into two groups according to their ages: Group A (children, ages 4–10 years) and Group B (adults, ages 14–39 years). In Group A, we observed significantly lower levels of complexes III and V in the cerebellum ( $p < 0.05$ ), of complex I in the frontal cortex ( $p < 0.05$ ), and of complexes II ( $p < 0.01$ ), III ( $p < 0.01$ ), and V ( $p < 0.05$ ) in the temporal cortex of children with autism as compared to age-matched control subjects, while none of the five ETC complexes was affected in the parietal and occipital cortices in subjects with autism. In the cerebellum and temporal cortex, no overlap was observed in the levels of these ETC complexes between subjects with autism and control subjects. In the frontal cortex of Group A, a lower level of ETC complexes was observed in a subset of autism cases, that is, 60% (3/5) for complexes I, II, and V, and 40% (2/5) for complexes III and IV. A striking observation was that the levels of ETC complexes were similar in adult subjects with autism and control subjects (Group B). A significant increase in the levels of lipid hydroperoxides, an oxidative stress marker, was also observed in the cerebellum and temporal cortex in the children with autism. These results suggest that the expression of ETC complexes is decreased in the cerebellum and the frontal and temporal regions of the brain in children with autism, which may lead to abnormal energy metabolism and oxidative stress. The deficits observed in the levels of ETC complexes in children with autism may readjust to normal levels by adulthood.

### Keywords

autism; electron transport chain complexes; energy; mitochondria; oxidative stress

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### Supporting information

Additional supporting information may be found in the online version of this article:

**Table S1.** Case history of autism and control brain samples.

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Autism is a complex pervasive developmental disorder that is characterized by impaired language, communication, and social skills, as well as by repetitive and stereotypic patterns of behavior, all occurring by the age of 3 years (Lord *et al.* 2000). It is a heterogeneous disorder, belonging to a group of neurodevelopmental disorders, known as the autism spectrum disorders (ASDs) that include Asperger syndrome and pervasive developmental disorder-not otherwise specified. According to a recent report from the Centers for Disease Control and Prevention, the prevalence of autism by the age of 8 years is 1 in 110 children (Rice 2009). The onset of autism is gradual in many children. However, functional regression has been reported in early childhood in some autism cases (Goldberg *et al.* 2003; Lord *et al.* 2004; Ozonoff *et al.* 2005; Hansen *et al.* 2008). Accumulating evidence supports a prenatal onset for developmental abnormalities leading to autism (Kolevzon *et al.* 2007; Kinney *et al.* 2008). Postmortem assessments of the brains of individuals with autism have unveiled early neurodevelopmental alterations, including reduced programmed cell death and/or increased cell proliferation, altered cell migration, abnormal cell differentiation with reduced neuronal size, and altered synaptogenesis (Bauman and Kemper 2005; Wegiel *et al.* 2009, 2010).

Mitochondria are central to many cellular functions, including the generation of energy in the form of ATP and the maintenance of intracellular calcium homeostasis. They are the primary source of free radicals, that is, reactive oxygen species (ROS) and trigger apoptosis (Cadenas and Davies 2000; Lenaz 2001; Szewczyk and Wojtczak 2002; Polster and Fiskum 2004). Neurons in particular rely on the mitochondria because of neurons' high levels of metabolism and subsequent need for energy. Mitochondria are localized in synapses, and alterations of the number, morphology, or function of synaptic mitochondria can be detrimental to synaptic transmission (Polster and Fiskum 2004). Extensive evidence suggests that mitochondrial dysfunction, oxidative stress, and reduced neurotransmission occur in the early stages of several major neurodegenerative diseases, such as Alzheimer's disease (Reddy 2008; Reddy and Beal 2008; Aliev *et al.* 2009; Wang *et al.* 2009), Parkinson's disease (Schapira *et al.* 1990; Navarro *et al.* 2009), Huntington disease (Gu *et al.* 1996), and amyotrophic lateral sclerosis (Wiedemann *et al.* 2002). Mitochondrial decay has also been suggested to be major contributor to aging (Ames 2004; Reddy 2008). In addition, mitochondrial dysfunction in the brain of some individuals with schizophrenia has been reported (Bubber *et al.* 2004). However, brain mitochondria have not yet been studied in autism, although altered energy metabolism as evidenced by alterations in peripheral markers, such as increased plasma lactate levels has been suggested in autism (Filipek *et al.* 2004; Correia *et al.* 2006).

Mitochondria are responsible for most of the energy production through oxidative phosphorylation, a process requiring the action of various respiratory enzyme complexes, the mitochondrial electron transport chain (ETC) located in the inner mitochondrial membrane (Szewczyk and Wojtczak 2002; Boekema and Braun 2007). Mitochondria produce ATP by generating a protons gradient (membrane potential) with the help of five ETC complexes, that is, complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *bc<sub>1</sub>* complex), complex IV (cytochrome *c* oxidase), and ATP synthase, also known as complex V, where the electron transport couples with translocation of protons from the mitochondrial matrix to the intermembrane space. The generated proton gradient is

used by ATP synthase to catalyze the formation of ATP by the phosphorylation of ADP (Scholes and Hinkle 1984; Bertram *et al.* 2006). The number of mitochondria per cell is roughly related to the energy demands of the cell. The brain has a high demand for energy, and neurons contain a large number of mitochondria. The ETC in mitochondria is also a prime mechanism for free radicals generation (Cadenas and Davies 2000; Lenaz 2001). The changes in the mitochondrial ETC have been suggested to be an important factor in the pathogenesis of several diseases, including neuropsychiatric (Rezin *et al.* 2009) and neurodegenerative disorders (Burchell *et al.* 2010; Moreira *et al.* 2010).

In this study, we compared the protein levels of various mitochondrial respiratory ETC complexes in different regions of the brain from subjects with autism and age-matched control subjects. Although children with autism showed a decrease in protein levels of ETC complexes in the cerebellum and the frontal and temporal cortices, no change was observed in the occipital and parietal cortices. Interestingly, when we analyzed the data as a function of age, children with autism (4–10 years of age) but not adults with autism (14–39 years of age) showed lower protein levels of brain ETC complexes, suggesting that developmental mitochondrial abnormalities resulting in mitochondrial dysfunction, oxidative stress, and abnormal energy metabolism may contribute to autistic phenotype.

## Materials and methods

### Materials

Samples of postmortem frozen brain regions, that is, the cerebellum, and cortices from the frontal, temporal, parietal, and occipital lobes ( $N = 7-8$  for different brain regions) from subjects with autism and age-matched control subjects were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Donors with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV, as confirmed by the Autism Diagnostic Interview-Revised. All brain samples were stored at  $-70^{\circ}\text{C}$ . This study was approved by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities. The case history (diagnosis, age, postmortem interval, and cause of death) for the subjects with autism and control subjects is summarized in Table S1.

### Preparation of brain homogenates

The tissue samples were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) in a Downs homogenizer with five strokes at  $4^{\circ}\text{C}$ . The protein concentration was assayed by bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA).

### Western blotting

The brain homogenates of subjects with autism and control subjects were mixed with loading buffer and boiled in a water bath for 5 min. Fifty micrograms of total protein of each sample was separated using a 12% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis and then transferred to a nitrocellulose membrane (0.45  $\mu\text{m}$ ; Bio-Rad Laboratories, Hercules, CA, USA) using 100 V for 40 min. The membrane was blocked with Tris-buffered saline containing 5% fat-free dried milk for 1 h at 22°C, and further incubated overnight at 4°C with mouse monoclonal OXPHOS antibody (dilution 1 : 1500; MitoSciences, Eugene, OR, USA) against mitochondrial ETC complexes I–V. The membrane was then washed with Tris-buffered saline-0.05% Tween 20 three times and incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1 : 5000; Thermo Scientific) for 45 min at 22°C. The membrane was washed again, and the immunoreactive proteins were visualized using the ECL substrate (Thermo Scientific). The levels of  $\beta$ -actin (the loading control) were determined by stripping and re-probing the membrane with anti- $\beta$ -actin antibody (dilution 1 : 20 000; Abcam, Cambridge, MA, USA). While the two top bands for ETC complexes II and V were clearly visible, other complexes were relatively faint on the same blot. Therefore, to enhance visualization of the remaining ETC complexes, the membrane was re-exposed for a longer period of time.

The film was scanned and the bands were analyzed by using Image J software (NIH, Bethesda, MD, USA). The densities of different mitochondrial ETC complexes and  $\beta$ -actin were estimated. The relative densities of the mitochondrial complexes versus  $\beta$ -actin in autism and control groups were compared by unpaired Student's *t*-test

### Measurement of lipid hydroperoxide (LOOH)

The levels of LOOH were measured in the brain homogenates, as described by Patsoukis and Georgiou 2007. The photometric assay of LOOH measurement is based on the reaction of  $\text{Fe}^{3+}$  with LOOH, which converts  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The reaction of  $\text{Fe}^{2+}$  with the reagent dye xylenol orange results in the formation of chromogenic product, which is measured at 560 nm.

## Results

The levels of different ETC complexes were measured in the cerebellum and the frontal, parietal, occipital, and temporal cortices of autism and age-matched controls by western blotting. The relative density of the bands of different mitochondrial complexes versus  $\beta$ -actin (loading control) is plotted as a histogram, and scattered plots show overall distribution of the data. Analysis of the data revealed lower levels of the ETC complexes in the cerebellum and the frontal and temporal cortices in the children with autism of ages 4–10 years than in age-matched controls, but not in autistic group of 14–39 years of age. None of the ETC complexes showed any difference in parietal and occipital cortices between subjects with autism and control subjects in any age group, suggesting that there are brain region-specific changes in mitochondrial ETC complexes in children with autism. Therefore, we divided the samples into two groups: Group A (children, 4–10 years) and Group B (adults, 14–39 years). The densitometric data of all ETC complexes normalized to  $\beta$ -actin are shown for all brain regions in Group A, Group B, and the entire group, that is, Group A + Group B. The scattered plot of samples is only shown when statistically significant changes in ETC complexes between autism and control groups were observed.

### Lower levels of ETC complexes III and V in the cerebellum of children with autism

Western blot analysis of the levels of different ETC complexes in the cerebellum of subjects with autism and age-matched control subjects is shown in Fig. 1(a) (Group A, age: 4–10 years) and Fig. 1(b) (Group B, age: 14–39 years). The relative densities of different ETC complexes normalized to that of  $\beta$ -actin (loading control) are presented in Fig. 1(c) (Group A), Fig. 1(e) (Group B), and Fig. 1(f) (Groups A + B). In Group A, significantly lower levels were observed for complex III [mean  $\pm$  SE =  $0.629 \pm 0.032$  (autism),  $0.899 \pm 0.067$  (control),  $p < 0.05$ ] and complex V [mean  $\pm$  SE =  $0.823 \pm 0.032$  (autism),  $1.154 \pm 0.105$  (control),  $p < 0.05$ ] in subjects with autism as compared with age-matched controls (Fig. 1c). Scattered plot of the data in Group A showed that there was no overlap for complexes III and V between subjects with autism and control subjects (Fig. 1d). A trend toward lower levels of complex II was also observed in subjects with autism compared to control subjects, but it was not significant (Fig. 1c), while the levels of complexes I and IV were similar between subjects with autism and control subjects. In adults, that is, Group B, there was no change in the levels of ETC complexes in subjects with autism compared with those in age-matched controls. However, a decrease in complex II was observed in 66% of subjects with autism (mean  $\pm$  SE =  $0.38 \pm 0.102$ ) compared with control subjects (mean  $\pm$  SE =  $0.626 \pm 0.139$ ), but it was not significant. When the data were analyzed for Group A + Group B, lower levels of complexes II, III, and V were observed in subjects with autism, but it was not significant (Fig. 1f).

### Lower levels of ETC complexes in the frontal cortex of children with autism

Western blot analysis of the levels of ETC complexes in the frontal cortex of subjects with autism and age-matched control subjects is shown in Fig. 2(a) for Group A, and Fig. 2(b) for Group B. Histogram analysis of the relative density of the data of ETC complexes is shown in Fig. 2(c) for Group A, Fig. 2(e) for Group B, and Fig. 2(f) for the entire Group A + B. When data in Group A were analyzed, a significant decrease in levels was observed for only complex I [(mean  $\pm$  SE =  $0.143 \pm 0.073$  (autism),  $0.395 \pm 0.044$  (control),  $p < 0.05$ )]; however, a general trend toward decreases in levels of the other complexes, that is, II–V was also observed (Fig. 2c). It was interesting to observe from the scattered plot that 60% (3/5) of complexes I, II, and V, and 40% (2/5) of complexes III and IV in the autism group had levels below the cutoff lower range for the control group, suggesting that a subset of autism cases has decreased levels of all ETC complexes in the frontal cortex.

In Group B, no change was observed in the levels of ETC complexes (Fig. 1e), except that a non-significant decrease was observed for complex III, where 66% of subjects with autism had decreased levels. When both Groups A and B were analyzed together (Fig. 2f), a general trend toward decreases in the levels of all ETC complexes was observed in subjects with autism, but it was not significant.

### Lower levels of ETC complexes II, III and V in the temporal cortex of children with autism

Western blot analysis of the levels of ETC complexes in the temporal cortex of subjects with autism and age-matched control subjects is shown in Fig. 3(a) (Group A) and Fig. 3(b) (Group B). Data analysis in Group A showed that the levels of complexes II, III, and V were significantly lower in subjects with autism as compared with age-matched control subjects

(Fig. 3c). The mean values  $\pm$  SE were as follows: for complex II in autism,  $0.425 \pm 0.082$ , in control,  $0.787 \pm 0.022$  ( $p < 0.01$ ); complex III in autism,  $0.710 \pm 0.008$ , and in control,  $0.972 \pm 0.038$  ( $p < 0.01$ ); and complex V in autism,  $0.813 \pm 0.083$ , and in control,  $1.147 \pm 0.048$  ( $p < 0.05$ ). Scattered plot analysis showed that there was no overlap in the levels of these ETC complexes between subjects with autism and control subjects. In contrast, no significant change in any of the ETC complexes was observed in Group B (Fig. 5f). When both Groups A and B were combined, only complex II was significantly decreased ( $p < 0.05$ ) in subjects with autism (mean  $\pm$  SE =  $0.474 \pm 0.057$ ) as compared to control subjects (mean  $\pm$  SE =  $0.706 \pm 0.053$ ) (Fig. 3f).

### **The levels of ETC complexes are not affected in parietal and occipital cortices of subjects with autism**

Western blots of ETC complexes in the parietal cortex (Fig. 4a and b) and occipital cortex (Fig. 5a and b) and histograms of relative densities (parietal cortex, Fig. 4c–e; occipital cortex, Fig. 5c–e) showed that the levels of ETC complexes are not affected in Group A as well as in Group B of subjects with autism as compared to age-matched control subjects. These results suggest that there is a brain region-specific decrease in the levels of ETC complexes in the cerebellum and the frontal and temporal cortices but not in the parietal and occipital cortices of subjects with autism. Because the parietal and occipital cortices were not affected in subjects with autism in comparison with control subjects, while the frontal and temporal cortices and the cerebellum from subjects with autism were affected, our results indirectly suggest that postmortem interval is not a contributing factor toward the observed brain mitochondrial abnormalities in autism.

### **Increased levels of LOOHs in specific brain regions in children with autism**

To assess whether changes in mitochondrial ETC in the children with autism also results in increased free radical generation and oxidative stress, we measured the levels of LOOH, a product of fatty acid oxidation, in the frontal, temporal, occipital and parietal cortices, and cerebellum from children with autism and age-matched controls (Fig. 6). The levels of LOOH were significantly increased in the cerebellum and temporal cortex of subjects with autism as compared with age-matched control subjects in Group A. An increase in the levels of LOOH was also observed in the frontal cortex in autism group, but it was not statistically significant. No change in the levels of LOOH was observed in the parietal and occipital cortices between autism and control groups.

## **Discussion**

Although the cause of autism remains elusive, it is considered a multifactorial disorder that is influenced by genetic, environmental, and immunological factors as well as increased vulnerability to oxidative stress (Chauhan and Chauhan 2006). In this study, we report two interesting observations: (i) brain region-specific changes occur in the levels of ETC complexes in the cerebellum and the frontal and temporal cortices but not in the parietal and occipital cortices in subjects with autism, and (ii) the changes above are observed only in young children with autism but not in adults with autism. We recently reported that the activities of  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase and  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase were also affected in the cerebellum

and frontal cortex, suggesting that the cerebellum and frontal cortex may have biochemical changes in autism (Ji *et al.* 2009). Mitochondria are vulnerable to a wide array of endogenous and exogenous factors, which appear to be linked by excessive production of free radicals. The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria (Cadenas and Davies 2000; Lenaz 2001). Mitochondria are not only the source of free radicals, but they are also the target of oxidative damage. In addition to producing more oxidants, damaged mitochondria are also vulnerable to oxidative stress. Increasing evidence from our and other groups suggests a role of oxidative stress in the development and clinical manifestation of autism (McGinnis 2004; Chauhan and Chauhan 2006). Levels of oxidative stress markers are increased in the blood (Chauhan *et al.* 2004; James *et al.* 2004; Zoroglu *et al.* 2004; Chauhan and Chauhan 2006), urine (Ming *et al.* 2005), and brains (Lopez-Hurtado and Prieto 2008; Evans *et al.* 2009; Muthaiyah *et al.* 2009; Sajdel-Sulkowska *et al.* 2009) of individuals with autism as compared with controls. In this study, increased levels of LOOH were also observed in the children with autism in same brain regions where mitochondrial ETC abnormalities were observed. As ETC in mitochondria is a prime source for ROS generation, these results also support the findings on mitochondrial dysfunction in children with autism.

Mitochondria play a central role in the energy-generating process through the transfer of electrons with the help of five ETC complexes and generation of a proton gradient in the inner membrane of the cell. Although the end product of the respiratory chain is water that is generated in a four-electron reduction of molecular oxygen by complex IV, a minor proportion of O<sub>2</sub> can be involved in the one-electron reduction processes generating ROS, in particular, superoxide anion radical ( $\cdot\text{O}_2^-$ ), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the extremely reactive hydroxyl radical (OH $\cdot$ ). Generation of ROS occurs mainly at complex III as a result of proton cycling between ubiquinone, cytochromes *b* and *c*1, and iron-sulfur protein (Sugioka *et al.* 1988). Some contribution of complex I to this process has also been found. Consequently, abnormalities in the levels of ETC complexes may be responsible for the observed oxidative stress in autism. The brain is highly vulnerable to oxidative stress, as it represents only 2% of the total body weight, but it accounts for 20% of all oxygen consumption, reflecting its high rate of metabolic activity (Juurlink and Paterson 1998; Shulman *et al.* 2004). Mitochondria have a crucial role in the supply of energy to the brain. Damaged ETC complexes compromise ATP synthesis and accelerate the generation of free radicals. Therefore, the mitochondrial ETC defects observed in the brains of young individuals with autism may have important, detrimental consequences on the function and plasticity of neurons in autism.

Mitochondrial diseases have been linked to poor growth, loss of muscle coordination, muscle weakness, developmental delays, learning disabilities, mental retardation, gastrointestinal disorders, neurological problems, seizures, and dementia (Read and Calnan 2000; Xu *et al.* 2005; Aliev *et al.* 2009). Depending on how severe the mitochondrial disorder is, the illness can range in severity from mild to fatal. It should be noted that some of the symptoms of mitochondrial diseases, such as learning disabilities, mental retardation, seizures, neurological problems, and gastrointestinal disturbances, are also present in a subset of individuals with autism.

Although this study is the first to report on brain mitochondrial abnormalities in autism, a few case reports of mitochondrial disorder have been reported in individuals with autism on the basis of blood analysis and/or muscle biopsy. These case reports include a child with autism with documented complex IV deficiency (Laszlo *et al.* 1994); a boy with autism with complex IV defect and a mtDNA G8363A mutation (Graf *et al.* 2000); two children with autism with deficiencies in several respiratory chain enzymes, including complexes I–III and coenzyme Q (Tsao and Mendell 2007); and five individuals with autism and mtDNA mutations or a mtDNA deletion (Pons *et al.* 2004). Anatomical and neuroradiographical studies of the brains of individuals with autism have also suggested that a disturbance of energy metabolism may be present (Lombard 1998; Chugani *et al.* 1999). <sup>31</sup>P-Magnetic resonance spectroscopy showed increased membrane degradation and decreased synthesis of ATP in autism (Minshew *et al.* 1993). In addition, carnitine deficiency in plasma, accompanied by elevations in lactate, alanine, and ammonia levels in autism, findings suggestive of mild mitochondrial dysfunction was reported in autism (Filipek *et al.* 2004). Another study also showed a high frequency of increased plasma lactate levels and increased lactate/pyruvate ratio in individuals with autism (Correia *et al.* 2006). Although the mechanism of hyperlactacidemia remains unknown, these case reports support dysfunction of mitochondrial oxidative phosphorylation in autism.

A population-based study in Portugal examining medical conditions in 120 children with autism found a disproportionately high prevalence (7%) of mitochondrial diseases in individuals with autism (Oliveira *et al.* 2005). However, these children did not have any known mtDNA mutations and/or deletions associated with known mitochondrial disorders. This report suggests that a substantial percentage of subgroups of autism may have a mitochondrial disorder.

The risk of sudden death of individuals who have inverted duplication of chromosome 15q (idic 15) is approximately 1% per year (Cleary 2009). This abnormality occurs in 1–5% of individuals with autism (Gillberg 1998; Schroer *et al.* 1998). Children with autism with a chromosome 15q11-q13 inverted duplication have been found to have motor delay, lethargy, severe hypotonia, and modest lactic acidosis. It is of interest to note that two children with autism and idic 15 showed mitochondrial hyperproliferation and complex III defect (Filipek *et al.* 2003), and two autism cases associated with sudden infant death syndrome showed mild mitochondrial hyperproliferation and a possible complex II defect (Gargus and Imtiaz 2008). These studies suggest that candidate gene loci for autism within the critical region may affect pathways influencing mitochondrial function (Filipek *et al.* 2003).

In regressive autism, children first show signs of normal social and language development through the first year of life but lose these developmental skills at 15–24 months and develop autistic behavior (Ozonoff *et al.* 2005). The rate of regressive autism varies from 15% to 62% of cases (Goldberg *et al.* 2003; Lord *et al.* 2004; Hansen *et al.* 2008). A recent study examined a group of 25 individuals with autism who also had confirmed mitochondrial disorders (Weissman *et al.* 2008). They reported that 40% of this group demonstrated unusual pattern of regression (multiple episodes, loss of motor skills, and regression after the age of 3). In this cohort, the deficiency of ETC complexes I and III was observed in 64% and 20% of individuals with autism, respectively, and two had a rare mtDNA mutation. Another



case report implicated mitochondrial dysfunction as a factor contributing to vaccine-related regression (Poling *et al.* 2006; Zecavati and Spence 2009). A recent report also suggests that fever in children with mitochondrial disease is a risk to autistic regression (Shoffner *et al.* 2010).

This study suggests that abnormalities in the mitochondrial ETC complex levels may be one of the factors in the etiology of autism. This will lead to oxidative stress and abnormal energy metabolism in autism. In our studies, deficiency of mitochondrial ETC complexes was observed in children with autism (ages 4–10 years) but not in adults with autism (14–39 years of age). Age also seems to play a critical role in determining brain growth in autism. Enlarged brain size (megalencephaly), particularly in the temporoparietal region, is the most consistent observation in young children with autism (Goldberg *et al.* 1999). The initial accelerated brain growth in young children is followed by abnormal slowness and growth arrest that results in normalization of brain size in late childhood and in adults (Hardan *et al.* 2001; Aylward *et al.* 2002; Courchesne 2004; Herbert 2005). Head circumference measurements have also shown increased brain volume in young children, later returning to normal volume. Thus, very large differences between children with autism and normal children are evident at early ages, but differences are not seen in adult cases (Aylward *et al.* 2002; Courchesne 2004). In addition, age-related changes in cerebellar nuclei and inferior olives have also been reported in autism (Palmen *et al.* 2004). The pattern of age-related changes in the severity of autism symptoms also suggests that causative factors determine both developmental and age-associated modifications. While age-related increases in the severity of autism symptoms have been reported among individuals with idiopathic 15 syndrome (Rineer *et al.* 1998), significant improvement of communication and social behaviors with increasing age has been reported in other autistic cohorts (Mesibov *et al.* 1989; Piven *et al.* 1996). Recent evidence suggests that 3–25% children with a previous diagnosis of ASD recover and show normal ranges of cognitive, adaptive, and social skills (Helt *et al.* 2008).

Neuropathological studies in autism suggest prenatal and postnatal developmental abnormalities in multiple regions of the brain, including the cerebellum, frontal and temporal cortices, cortical white matter, amygdala, and brainstem (particularly the olivary nuclei) (Palmen *et al.* 2004; Bauman and Kemper 2005; Pickett and London 2005; Schmitz and Rezaie 2008; Wegiel *et al.* 2009, 2010). There is substantial evidence from neuroimaging studies that dysfunctions in the cerebellum and possibly the temporal lobe and association cortex result in autism symptoms. Loss of Purkinje and granule cells has been reported throughout the cerebellar hemispheres in autism (Bauman and Kemper 1985, 2005; Kern 2003; Casanova 2007). Alterations in neuronal size, density, and dendritic branching in the cerebellum and limbic structures (hippocampus and amygdala) have also been reported in autism.

The prevalence rate of mitochondrial disease is about one in 5000–10 000 children (Skladal *et al.* 2003; Schaefer *et al.* 2004). In contrast, the prevalence rate for autism is 1 in 110 children (Rice 2009). As we observed a high percentage of changes in complexes I–III, and V in the cerebellum and frontal and temporal cortices of individuals with autism, it seems that autism is associated with mitochondrial dysfunction, although clinical symptoms of mitochondrial disease may be lacking. Therefore, mitochondrial dysfunctions rather than

mitochondrial disorders may be more relevant in autism. The clinical diagnosis of mitochondrial disease is often made with biochemical analysis of lactate, pyruvate, and alanine in blood, urine, or cerebrospinal fluid. However, the analysis of biochemical metabolites to diagnose mitochondrial disease may not be sufficient, as these analyses seem to be frequently normal, even in some severe cases of the disease. The clinical symptoms of mitochondrial disease are increased when ASD has comorbidity, such as hypotonia and motor delay, fatigue, metabolic abnormalities, and epilepsy (Fillano *et al.* 2002). The genetics of autism is complex, with the involvement of multiple genes. However, no gene has been identified that follows the typical Mendelian laws of inheritance. ASDs may have mild changes in the levels of ETC complexes that may or may not be related to a gene mutation. The mild form of mitochondrial abnormalities observed in autism may also be linked to other abnormalities such as the excessive  $\text{Ca}^{2+}$  observed in the mitochondria in autism. Excessive levels of  $\text{Ca}^{2+}$  in the mitochondria can affect the mitochondrial metabolism and increase the oxidative stress in the brains of individuals with autism (Palmieri *et al.* 2010).

The mechanism by which mitochondrial dysfunction may occur and affect development of autism is not entirely clear. It is possible that in comparison with classical mitochondrial disease, mitochondrial dysfunction may show less severe symptoms and may not show the classical mitochondrial pathology on muscle biopsy (Lombard 1998). Further research with larger sample sizes is needed to determine the association between mitochondrial dysfunction and severity, clinical phenotypes, regression, and/or idic 15 in autism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used

<b>ASDs</b>	autism spectrum disorders
<b>ETC</b>	electron transport chain
<b>LOOH</b>	lipid hydroperoxide
<b>ROS</b>	reactive oxygen species

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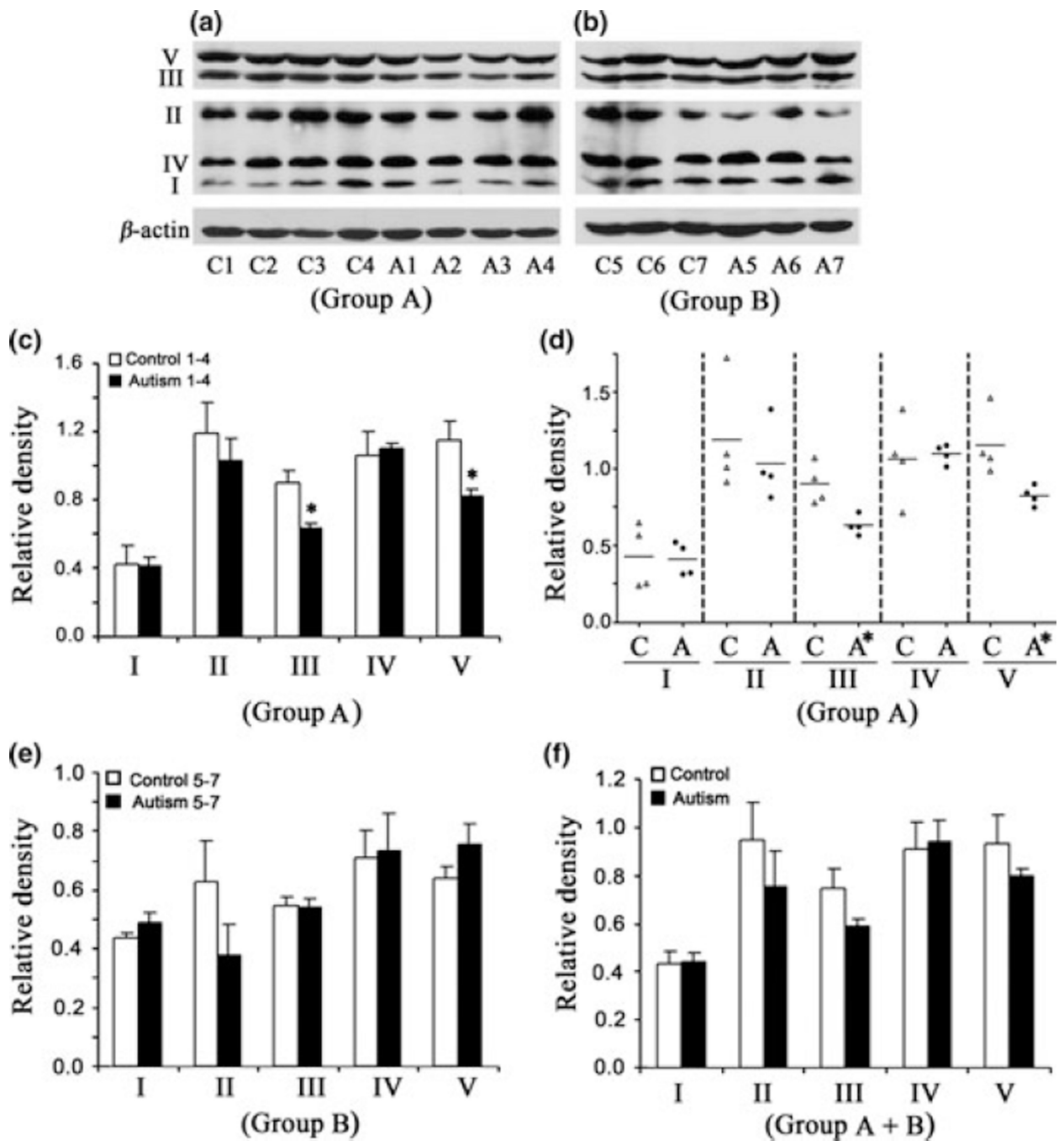
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**Fig. 1.** Electron transport chain (ETC) complexes in the cerebellum from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1-A4 for subjects with autism, and C1-C4 for control subjects (a), whereas Group B samples were A5-A7 for subjects with autism, and C5-C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to  $\beta$ -actin are shown in (c) (Group A), (e)

(Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). \* $p < 0.05$ , unpaired  $t$ -test.

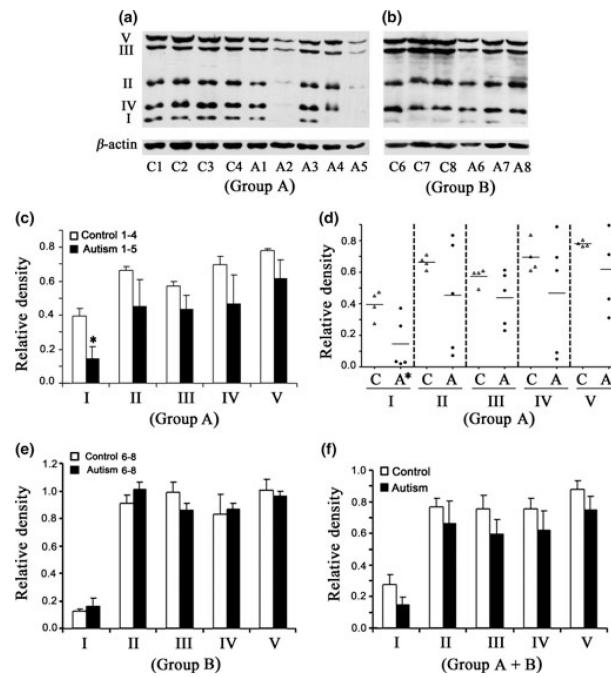
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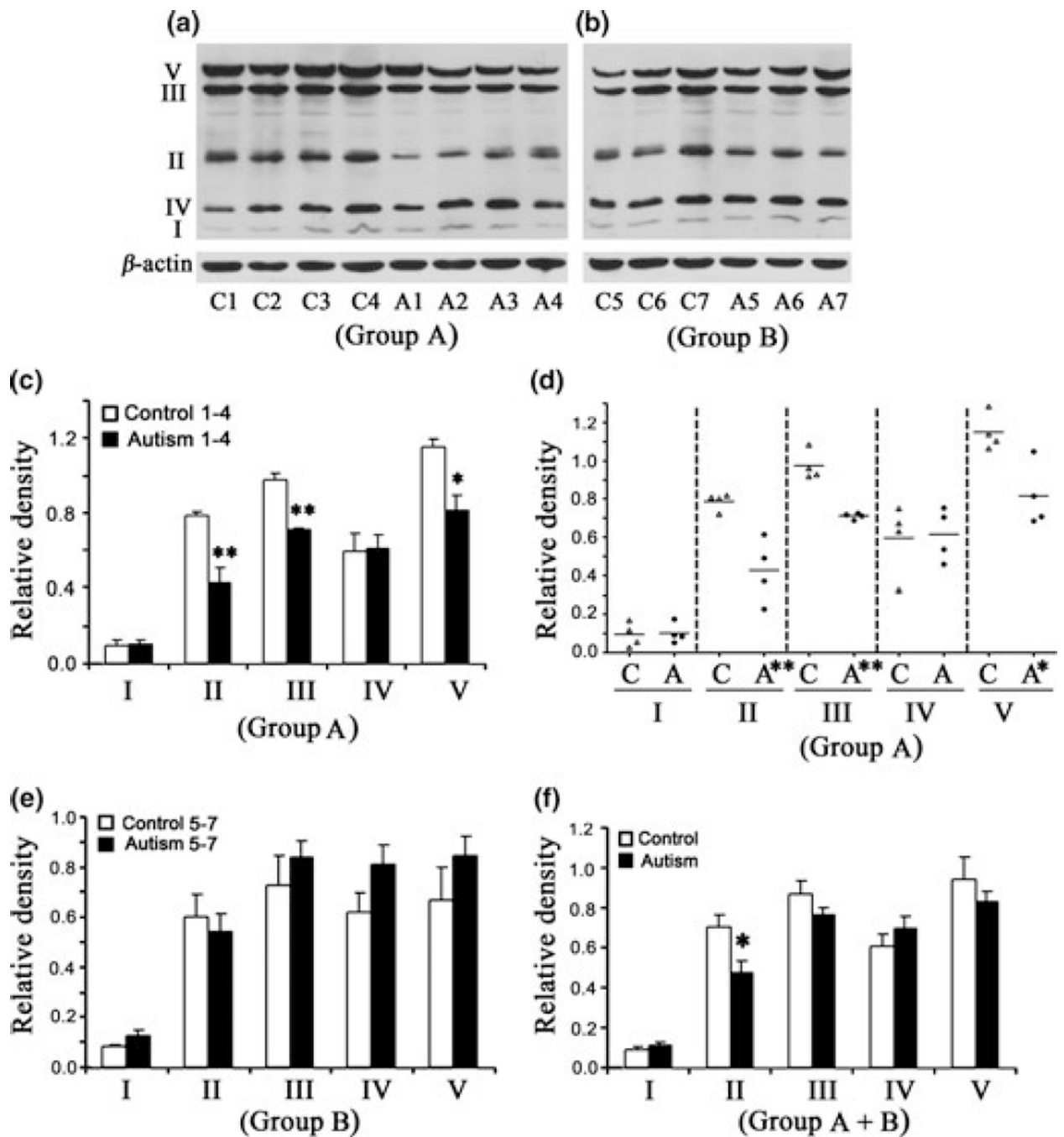
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**Fig. 2.**

Electron transport chain (ETC) complexes in the frontal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1-A5 for subjects with autism, and C1-C4 for control subjects (a), whereas Group B samples were A6-A8 for subjects with autism, and C5-C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to  $\beta$ -actin are shown in (c) (Group A), (e) (Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). \* $p < 0.05$ , unpaired  $t$ -test.



**Fig. 3.** Electron transport chain (ETC) complexes in the temporal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1-A4 for subjects with autism, and C1-C4 for control subjects (a), whereas Group B samples were A5-A7 for subjects with autism, and C5-C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to  $\beta$ -actin are shown in (c) (Group

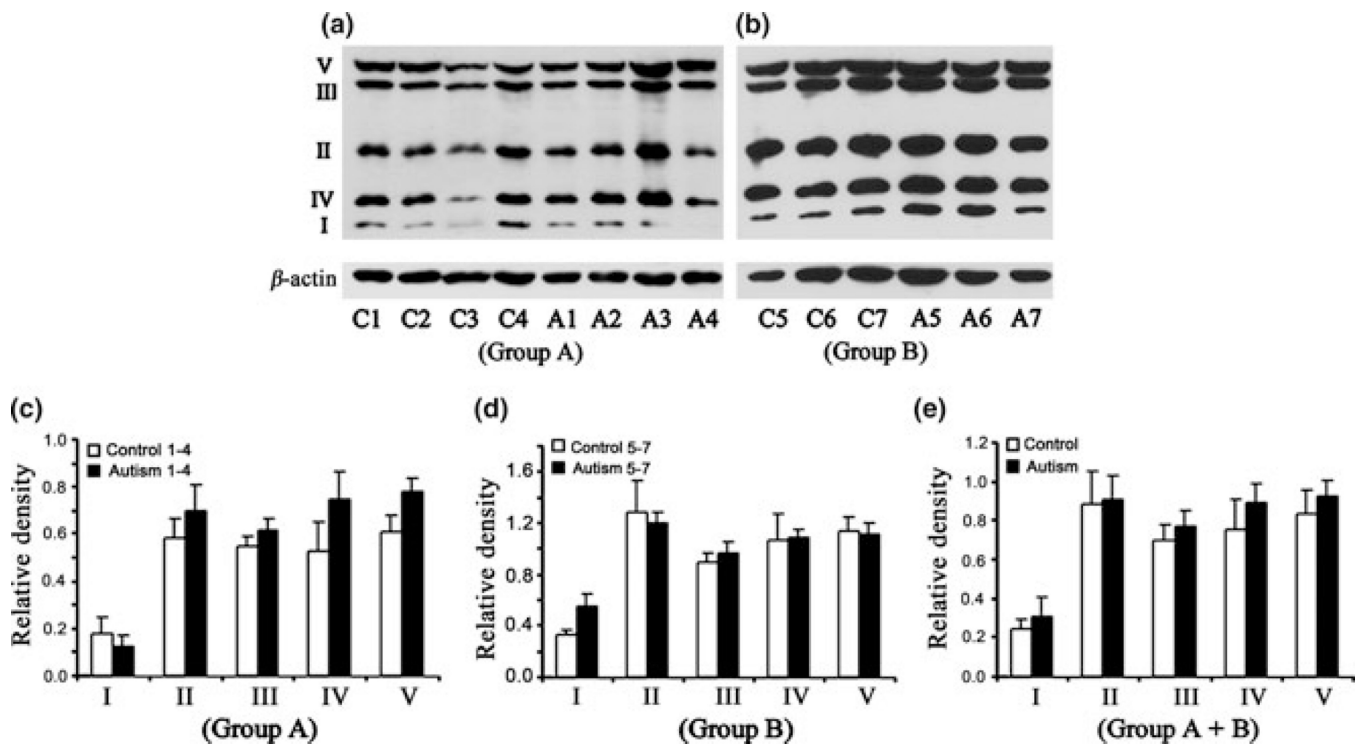
A), (e) (Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). \* $p < 0.05$ , \*\* $p < 0.01$ , unpaired  $t$ -test.

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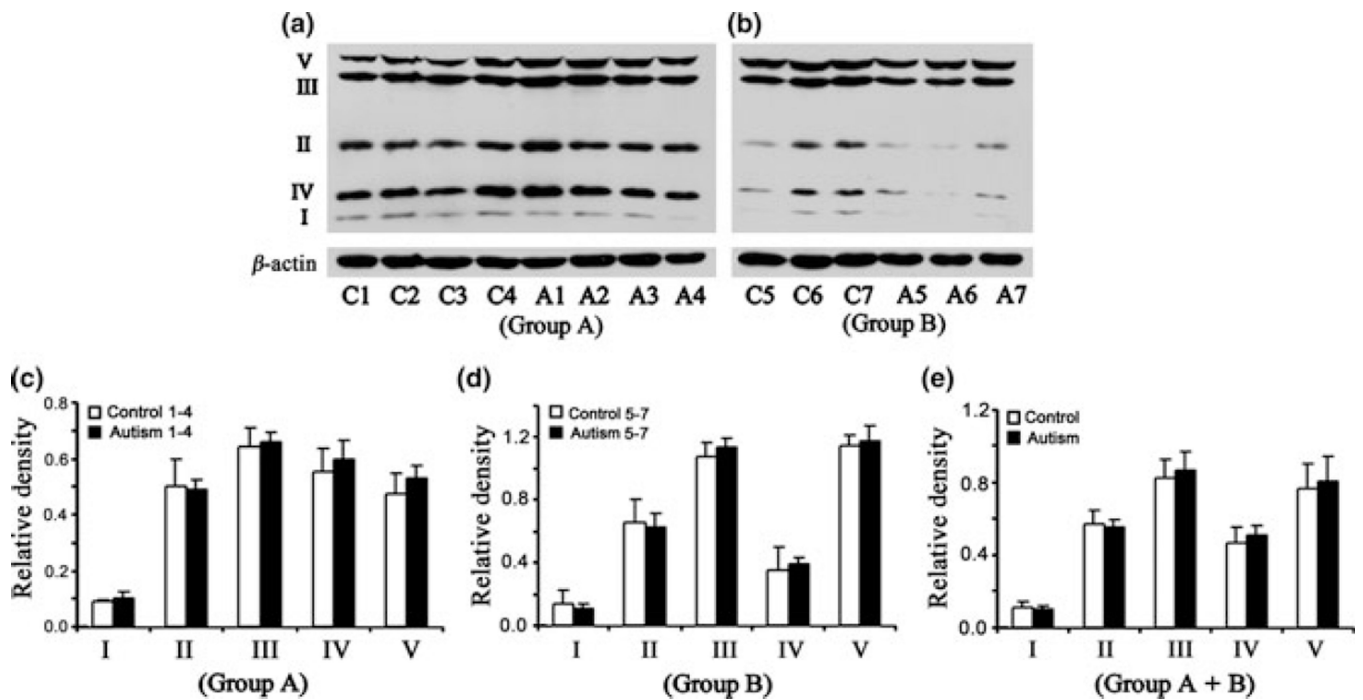
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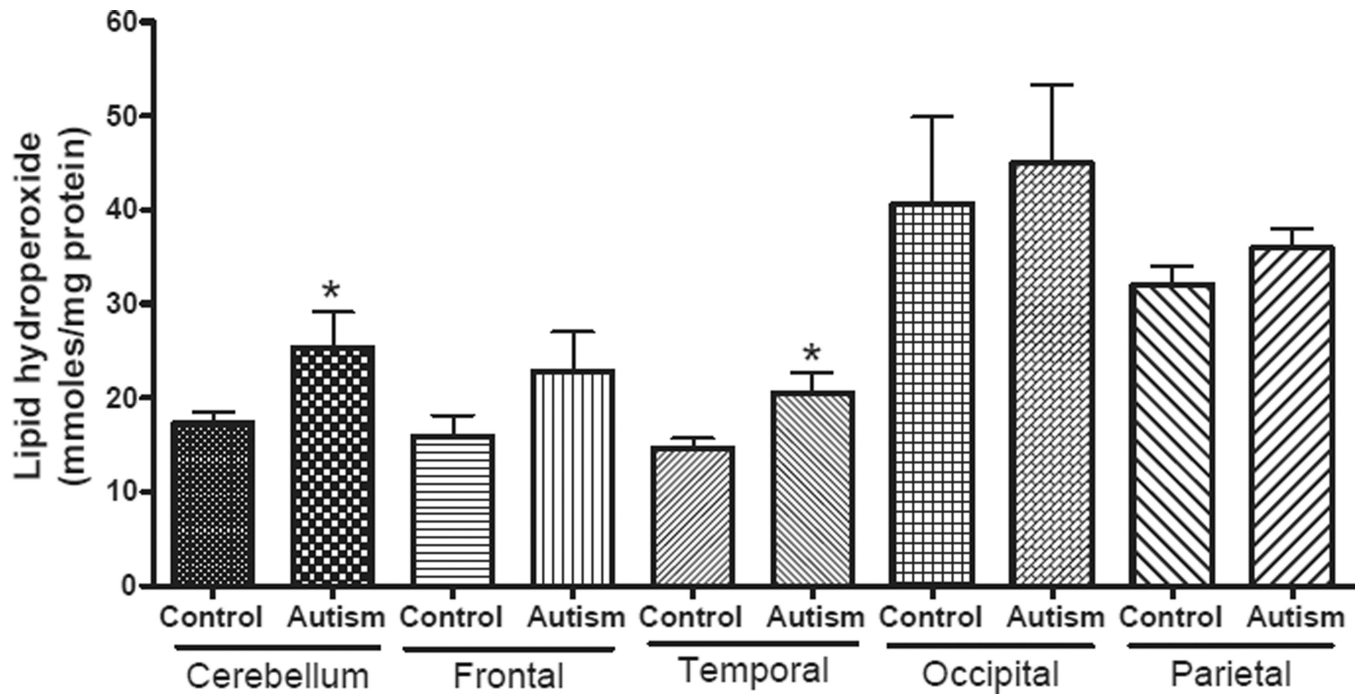
**Fig. 4.**

Electron transport chain (ETC) complexes in the parietal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1-A4 for subjects with autism, and C1-C4 for control subjects (a), whereas Group B samples were A5-A7 for subjects with autism and C5-C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to  $\beta$ -actin are shown in (c) (Group A), (d) (Group B), and (e) (combined Groups A + B).



**Fig. 5.**

Electron transport chain (ETC) complexes in the occipital cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1-A4 for subjects with autism, and C1-C4 for control subjects (a), whereas group B samples were A5-A7 for subjects with autism and C5-C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to  $\beta$ -actin are shown in (c) (Group A), (d) (Group B), and (e) (combined Groups A + B).



**Fig. 6.**

Levels of lipid hydroperoxides in different regions of brain from subjects with autism and age-matched controls in Group A (age: 4–10 years). Lipid hydroperoxides were measured in the brain homogenates from frontal, temporal, occipital and parietal cortices, and cerebellum of subjects with autism and age-matched controls in Group A. The data represent mean  $\pm$  SE. \* $p < 0.05$ , unpaired  $t$ -test.