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14. ABSTRACT

Brain tissue is highly heterogeneous with different functions localized in specific areas. Our results suggest that there is increased oxidative damage coupled with reduced glutathione antioxidant capacity in the selective regions of the brain in the subjects with autism. These changes in autism were specific to the frontal cortex, temporal cortex and cerebellum, while parietal and occipital cortices were not affected. Free radicals and energy (ATP) are generated by the mitochondrial in the cell with the help of mitochondrial electron transport chain (ETC) complexes. We have also reported brain region-specific deficit in mitochondrial ETC complexes in children.with autism. Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders. The complexity of ASDs is further increased because some affected individuals fall in the sub-group of regressive autism. We report here that individuals with regressive autism have decreased activities of protein kinases, i.e. protein kinase A (PKA) and protein kinase C (PKC) in the frontal cortex of the brain. Such changes were not observed in individuals with non-regressive autism. These results suggest that abnormal cellular signaling in the frontal lobe of the brain may be associated with regression in autism.

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Oxidative stress in autistic brain, free radicals, mitochondria, energy, protein kinases, cell signaling

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Table of Contents

Page

SUBPROJECT 3

Oxidative damage and inflammation in the brains of autistic subjects: Correlation with severity and phenotypes.

PI: Abha Chauhan, Ph.D.

INTRODUCTION

Autism is a heterogeneous, behaviorally defined neurodevelopmental disorder. There is limited knowledge of the causative factors and secondary abnormalities in biochemical pathways in autism. While the cause of autism remains elusive, autism is considered a multifactorial disorder that is influenced by genetic and environmental factors. Accumulating evidence suggests that oxidative stress may provide a link between susceptibility genes and pre- and post-natal environmental risk agents in the pathophysiology of autism [1-3]. Under normal conditions**,** a dynamic equilibrium exists between the production of free radicals, i.e. reactive oxygen species (ROS) and the anti-oxidant capacity of the cell. These ROS are highly toxic, and if not removed or neutralized, they react with lipids, proteins and nucleic acids and damage membrane properties and cellular functions. Glutathione (GSH) is the most important endogenous antioxidant in human tissues, which neutralizes ROS, and participates in detoxification and elimination of environmental toxins. Due to the lack of glutathione-producing capacity by neurons, the brain has a limited capacity to detoxify ROS. Therefore, neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and, as a result, they are most susceptible to oxidative stress. Oxidative stress is known to be associated with premature aging of cells and can lead to inflammation, damaged cell membranes, autoimmunity and cell death. The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement and high amounts of unsaturated lipids and iron [4]. The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons [5].

Extensive evidence suggests the presence of oxidative stress in peripheral tissues in children with autism [1, 3]. We have reported that levels of malonyldialdehyde, a marker of lipid peroxidation, are increased in the plasma from children with autism [6]. Other studies on erythrocytes and urine samples have also indicated increased levels of lipid peroxidation markers in autism, thus confirming an increased oxidative stress in autism [7, 8]. Brain tissue is highly heterogeneous with specific functions localized in specific areas of brain. The studies in this project with postmortem brain tissues have shown elevated levels of markers of oxidative damage, coupled with reduced antioxidant status in the cerebellum, frontal and temporal cortex of the brain of individuals with autism as compared to age-matched control subjects [9-12].

Mitochondria are the primary source of free radicals, and are central to many cellular functions including the generation of ATP (energy). They also trigger apoptosis, i.e. cell death. Neurons in particular rely on mitochondria because of their high levels of activity and subsequent need for energy. The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria [13]. Electron transport chain (ETC) in mitochondrion is a prime site for free radicals generation. Mitochondria generate ATP by generation of protons gradient (membrane potential) with the help of five ETC complexes. The changes in the mitochondrial ETC have been reported in several neurodegenerative disorders.

Recent evidence also suggests increased prevalence of mitochondrial dysfunction in autism [3, 14]. Our studies in this project have indicated brain region-specific deficit of mitochondrial ETC complexes in autism [9].

Protein kinases are known to play important roles in cellular signaling pathways and are involved in brain development. Protein kinase A (PKA) is a cyclic adenosine monophosphate (cAMP)–dependent protein kinase that is involved in cognitive functions and memory formation. Protein Kinase C (PKC), a ubiquitous phospholipid-dependent serine/threonine kinase, is another G-protein-coupled receptor-mediated kinase. PKC is known to be involved in signal transduction associated with the control of brain functions, such as ion channel regulation, receptor modulation, neurotransmitters release, synaptic potentiation/depression, and neuronal survival. It also plays crucial roles in cell proliferation, differentiation and apoptosis. In this project, we have examined the activities of PKA and PKC in the brain samples from autism and control subjects [15, 16].

BODY

In our study, the postmortem frozen brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex from autistic subjects with age range of 4 to 39 yrs from subjects with autism and age-matched control subjects were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland.

Increased oxidative damage in the frontal cortex, temporal cortex and cerebellum in autism. We observed brain region-specific increased levels of lipid hydroperoxide [9], a product of fatty acid oxidation; of malonyldialdehyde [10], an end-product of lipid peroxidation; of 8-hydroxy-2$^{'}$-deoxyguanosine (8-OH-dG) [11], a marker of oxidative DNA damage; and of protein carbonyl [12], a marker of protein oxidation in autism. Other groups have also reported elevated expression of carboxyethyl pyrrole [17], a marker of lipid-derived oxidative protein modification, and of 3-nitrotyrosine [18], a marker of protein nitration, in postmortem brain samples from autistic subjects.

Reduced antioxidant capacity in the brain of autistic subjects. In order to study antioxidant status of brain in autism, we examined the concentrations of glutathione (GSH, reduced form; and GSSG, oxidized form) and the redox ratio of GSH to GSSG (marker of oxidative stress) in different regions of brains from autistic subjects and age-matched control subjects [19, Appendix 1]. We have recently reported a decrease in GSH, an increase in its oxidized disulfide form (GSSG) and a decrease in the redox ratio of GSH/GSSG in the cerebellum and temporal cortex of individuals with autism, suggesting a glutathione redox imbalance in autism [19, Appendix 1]. These findings indicate that autism is associated with deficits in glutathione antioxidant defense in selective regions of the brain.

Mitochondrial dysfunction in autism. Since mitochondria play important roles in the generation of free radicals and ATP formation, we studied the levels of mitochondrial ETC complexes, i.e., complexes I, II, III, IV, and V, in brain tissue samples from the cerebellum and the frontal, parietal, occipital, and temporal cortices of autism and age-matched control subjects

[9]. We have reported brain region-specific deficit in mitochondrial ETC complexes in autism [9].

Increased activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase. Since increased oxidative stress in autism can affect the activities of membrane-bound enzymes, such as Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase that are known to maintain intracellular gradients of ions essential for signal transduction, we also studied whether oxidative stress can affect the activities of these enzymes in different brain regions of autistic subjects. In the cerebellum and frontal cortex of individuals with autism, we reported increased activities of Na^+/K^+- ATPase and Ca^{2+} /Mg^{2+}-ATPase [20].

Reduced activity of protein kinases in the frontal cortex of subjects with regressive autism: Relationship with developmental abnormalities. In regressive autism, affected children first show signs of normal social and language development but eventually lose these skills and develop autistic behavior. The underlying mechanism for regression in autism is not known. Protein kinases are essential for G-protein-coupled receptor-mediated signal transduction, and are involved in neuronal functions, gene expression, memory, and cell differentiation. Recently, we reported decreased activity of protein kinase A (PKA) in the frontal cortex of subjects with regressive autism [15, Appendix 2]. In the present study, we analyzed the activity of protein kinase C (PKC) in the cerebellum and different regions of cerebral cortex from subjects with regressive autism, autistic subjects without clinical history of regression, and age-matched control subjects [16, Appendix 3]. In the frontal cortex of subjects with regressive autism, PKC activity was significantly decreased by 57.1% as compared to age-matched control subjects ($p = 0.0085$), and by 65.8% as compared to non-regressed autistic subjects ($p = 0.0048$) (Fig. 1). PKC activity was unaffected in the temporal, parietal and occipital cortices, and in the cerebellum in both autism groups, i.e., regressive and non-regressed autism as compared to control subjects (Fig. 1,2). These results suggest brain region-specific alteration of PKC activity in the frontal cortex of subjects with regressive autism. Further studies showed a negative correlation between PKC activity and restrictive, repetitive and stereotyped pattern of behavior ($r= –0.084$, $p = 0.0363$) in autistic individuals, suggesting involvement of PKC in behavioral abnormalities in autism (Fig. 3). These findings suggest that regression in autism may be attributed, in part, to alterations in G-protein-coupled receptor-mediated signal transduction involving PKA and PKC in the frontal cortex.

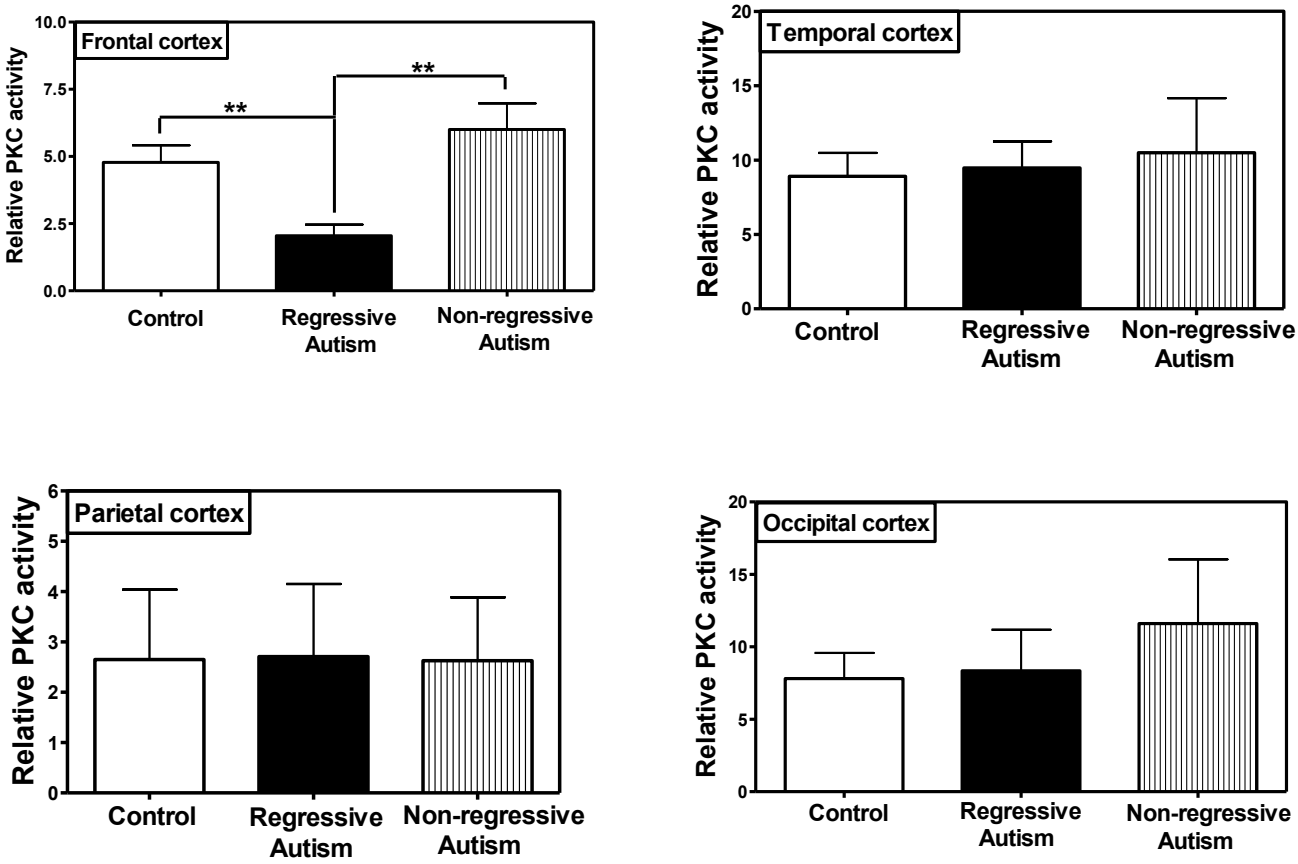

Fig. 1. Protein kinase C activity in different regions of cerebral cortex, i.e., frontal, temporal, occipital and parietal cortex from subjects with regressive autism, non-regressed autism and their age-matched controls. The mean absorbance ($x10^3$) of samples was divided by the quantity of total protein (μg) used per assay, and the data is represented as relative PKC activity. **$p < 0.01$ as compared to control and non-regressed autism groups.

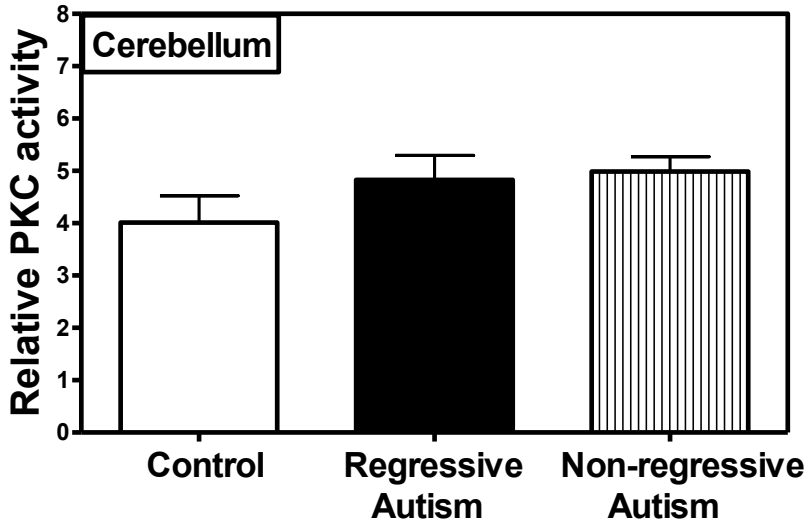

Fig. 2. Protein kinase C activity in the cerebellum from subjects with regressive autism, non-regressed autism and their age-matched control subjects. The mean absorbance ($x10^3$) of samples was divided by the quantity of total protein (μg) used per assay, and the data is represented as relative PKC activity.

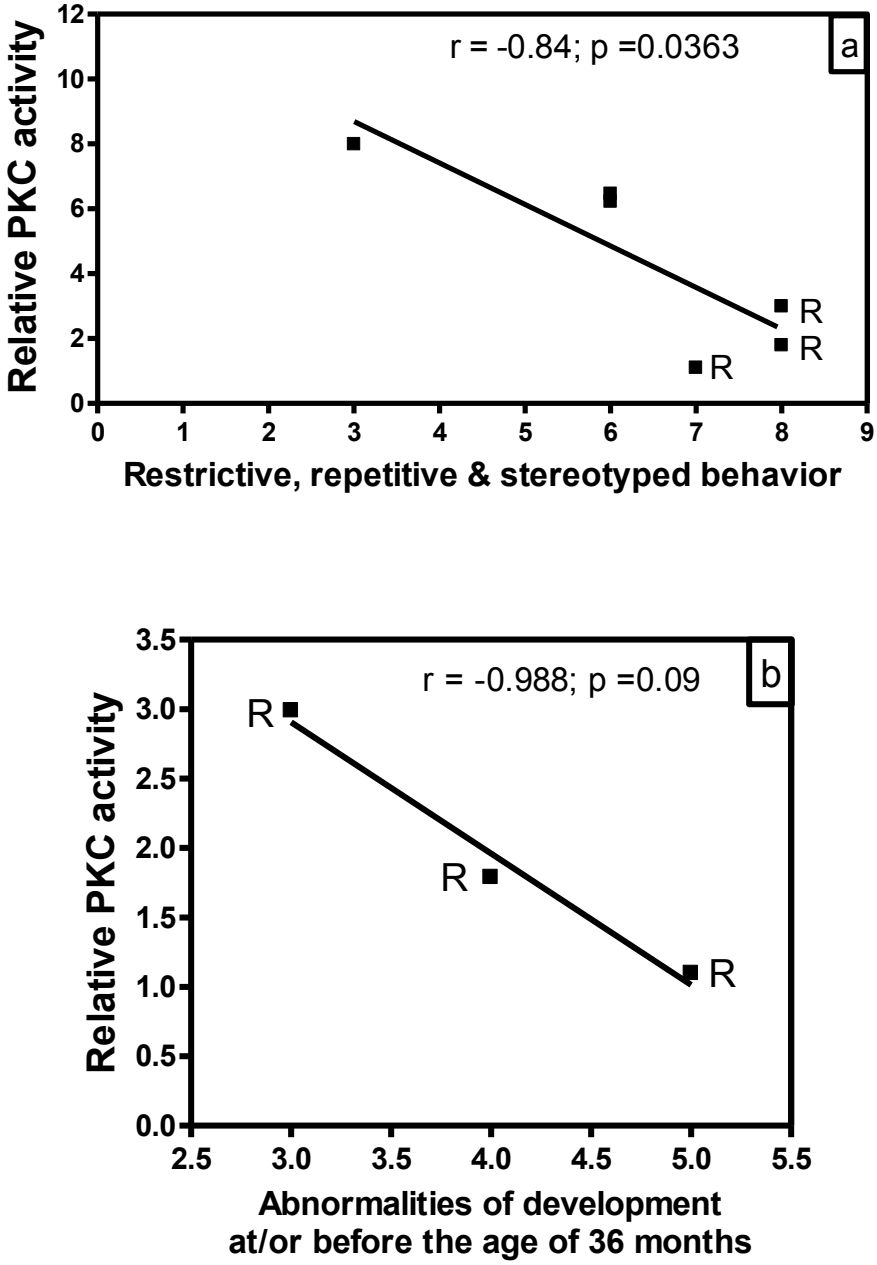

Fig. 3. Relationship between PKC activity of frontal cortex and Autism Diagnostic Interview Revised (ADI-R) test scores in subjects with autism. PKC activity was plotted against individual ADI-R scores for (a) restricted, repetitive and stereotyped patterns of behavior, and (b) abnormalities of development evident before the age of 36 months. R represents subjects with regressive autism.

KEY RESEARCH ACCOMPLISHMENTS

1. There is increased oxidative damage as evidenced by increase in lipid peroxidation, protein oxidation and DNA oxidation in the cerebellum, frontal cortex and temporal cortex of the brain in autism [9-12]. Oxidative stress is brain region-specific in autism, and was not observed in occipital and parietal cortex.

2. Glutathione antioxidant capacity is reduced in the cerebellum and temporal cortex in autism [19, Appendix 1].

3. There is a brain region–specific decrease in the levels of mitochondrial electron transport chain complexes in the cerebellum and in the frontal and temporal cortices but not in the parietal and occipital cortices of subjects with autism [9]. These mitochondrial abnormalities are observed only in young children with autism but not in adults with autism. The abnormalities in the mitochondrial ETC complex levels resulting in disruption of mitochondrial function may be one of the factors in the etiology of autism. This will lead to increased free radical generation, oxidative stress and abnormal energy metabolism in autism.

4. The activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase (membrane-bound enzymes) were significantly increased in the cerebellum in the autistic samples compared with their age-matched controls. The activity of Na^+/K^+-ATPase but not Ca^{2+}/Mg^{2+}-ATPase was also significantly increased in the frontal cortex of the autistic samples as compared to the age-matched controls [20]. In contrast, in other regions, i.e., the temporal, parietal and occipital cortices, the activities of these enzymes were similar in autism and control groups.

5. Individuals with regressive autism have decreased activities of PKA and PKC in the frontal cortex of the brain [15, Appendix 2; 16, Appendix 3]. Such changes were not observed in other brain regions of individuals with regressive autism, or in the frontal cortex and other brain regions of individuals with non-regressive autism. These results suggest that alterations in PKA activity and PKA expression are specific to the frontal lobe in regressive autism.

Our results suggest mitochondrial dysfunction, increased oxidative damage coupled with reduced antioxidant status in the specific regions of brain i.e., cerebellum, frontal and temporal cortex of autistic individuals compared with brain samples from age-matched control subjects. Our results also suggest altered activities of enzymes involved in cellular signaling such as Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase, PKA and PKC in specific brain regions in autism Frontal cortex may be the region of the brain involved in regressive autism, where abnormalities such as decreased activity of PKA and PKC can affect the signal transduction.

REPORTABLE OUTCOMES

Abstracts

1. Chauhan, A. (**Keynote Speaker).** Oxidative stress and mitochondrial dysfunction in autism: Impact of genetic and environmental factors. International Conference on Neurology and Therapeutics. J. Neurol. Neurophysiol. 3(2): 27 (May 14^{-16}, 2012).
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3. Chauhan, A., Audhya,T. and Chauhan, V.. Glutathione redox imbalance and increased DNA oxidation in specific brain regions in autism. J. Neurochem. 118 (Suppl.1), 217 (2011).

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9. Chauhan, A. and Chauhan, V. Brain Oxidative Stress and Mitochondrial Abnormalities in Autism (Review). In: Consensus Paper: Pathological role of the cerebellum in autism (Fatemi S.H. et al.). Cerebellum 11: 777-807 (2012).
10. Chauhan, A., Gu, F. and Chauhan, V. Mitochondrial respiratory chain defects in autism and other neurodevelopmental disorders Special Issue: Mitochondrial dysfunction associated with neurodevelopmental disorders. J. Pediatric Biochem. (review; in press)

News Release of above publications # 3 and 4

1. **News release of our publication in J. Neurochemistry (**Chauhan et al. Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism). **by Simons Foundation Autism Research Initiative (March 17, 2011)**

   https://sfari.org/news-and-commentary/open-article/-/asset_publisher/6Tog/content/mitochondrial-function-disrupted-in-children-with-autism?redirect=%2Fnews-and-commentary%2Fall

2. **Our article in J. Neurochemistry (**Chauhan et al. Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism) was featured as **key scientific article by Global Medical Discovery**

   http://globalmedicaldiscovery.com/key-scientific-articles/brain-region-specific-deficit-in-mitochondrial-electron-transport-chain-complexes-in-children-with-autism/.

3. **News release** (Molecular mechanisms: Pathway linked to regressive autism) by **Simons Foundation Autism Research Initiative** (Oct 12, 2011) for **our publication in PLoS One (**Brain region–specific decrease in the activity and expression of protein kinase A in the frontal cortex of regressive autism).

   http://sfari.org/news-and-opinion/in-brief/2011/molecular-mechanisms-pathway-linked-to- regressive-autism

4. Above article was also covered in the **press release** (New biochemical findings might explain why children with regressive autism lose skills) by **Decoded Science** (Oct 21, 2011**)**.
   http://www.decodedscience.com

CONCLUSIONS

Brain is a heterogeneous organ where specific functions are attributed to specific regions. Our results suggest that autism is associated with mitochondrial dysfunction and increased oxidative stress in the brain, which differentially affects selective regions of the brain, i.e. cerebellum, frontal cortex and temporal cortex in autism. We have also reported brain region-specific increased activities of membrane-bound enzymes, such as Na^{+}/K^{+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase that are known to maintain intracellular gradients of ions essential for signal transduction Our results also suggest lower activities of PKA and PKC in the frontal lobe of subjects with regressive autism, which will lead to abnormal cellular signaling. Increased oxidative damage may also lead to inflammation because oxidative stress serves as a major upstream component in the signaling cascade involved in activation of redox-sensitive transcription factors and pro-inflammatory gene expression resulting in an inflammatory response.

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APPENDICES

1. Chauhan, A, Audhya, T., and Chauhan, V. Brain region-specific glutathione redox imbalance in autism. Neurochem. Res. 37: 1681-1689 (2012).
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ORIGINAL PAPER

Brain Region-Specific Glutathione Redox Imbalance in Autism

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Abstract Autism is a heterogeneous, behaviorally defined neurodevelopmental disorder. Recently, we reported a brain region–specific increase in lipid peroxidation, and deficits in mitochondrial electron transport chain complexes in autism, suggesting the role of oxidative stress and mitochondrial dysfunction in the pathophysiology of autism. However, the antioxidant status of the brain is not known in autism. Glutathione is a major endogenous antioxidant that plays a crucial role in protecting cells from exogenous and endogenous toxins, particularly in the central nervous system. The present study examines the concentrations of glutathione (GSH, reduced form; and GSSG, oxidized form) and the redox ratio of GSH to GSSG (marker of oxidative stress) in different regions of brains from autistic subjects and age-matched control subjects. In the cerebellum and temporal cortex from subjects with autism, GSH levels were significantly decreased by 34.2 and 44.6 %, with a concomitant increase in the levels of GSSG by 38.2 and 45.5 %, respectively, as compared to the control group. There was also a significant decrease in the levels of total GSH (tGSH) by 32.9 % in the cerebellum, and by 43.1 % in the temporal cortex of subjects with autism. In contrast, there was no significant change in GSH, GSSG and tGSH levels in the frontal, parietal and occipital cortices in autism versus control group. The redox ratio of GSH to GSSG was also significantly decreased by 52.8 % in the cerebellum and by 60.8 % in the temporal cortex of subjects with autism, suggesting glutathione redox imbalance in the brain of individuals with autism. These findings indicate that autism is associated with deficits in glutathione antioxidant defense in selective regions of the brain. We suggest that disturbances in brain glutathione homeostasis may contribute to oxidative stress, immune dysfunction and apoptosis, particularly in the cerebellum and temporal lobe, and may lead to neurodevelopmental abnormalities in autism.

Keywords Autism · Brain · Glutathione · Neurodevelopment · Oxidative stress · Redox

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Introduction

Autism is a severe neurodevelopmental disorder characterized by deficits in social interaction; impairments in verbal and nonverbal communication; and restricted, repetitive and stereotyped patterns of behavior [1]. Autism belongs to a group of neurodevelopmental disorders known as autism spectrum disorders (ASDs), which include pervasive developmental disorder—not otherwise specified (PPD-NOS) and Asperger disorder. According to the Centers for Disease Control and Prevention, 1 in 110 children in the United States is diagnosed with ASDs [2].

Autism is a heterogeneous disorder, both etiologically and phenotypically. While the cause of autism remains elusive, autism is considered a multi-factorial disorder that is influenced by genetic, epigenetic, environmental and immunological factors [3, 4]. Accumulating evidence suggests that oxidative stress may be a common feature in

autism through which environmental factors exert their deleterious effects, which may be further exacerbated by the interaction of genetically susceptible alleles [3–6]. Several studies suggest that inflammatory phenomena, immune dysregulation and certain autoimmune risk factors may also contribute to the development and pathogenesis of autism [3, 7–9].

The brain is highly vulnerable to oxidative stress as a result of its limited antioxidant capacity, high energy requirement and high amounts of unsaturated lipids and iron [10]. Antioxidants, particularly glutathione, are essential for neuronal survival during the early critical period [11, 12]. Glutathione exists in the thiol-reduced form (GSH) and disulfide-oxidized form (GSSG). GSH is the most important endogenous antioxidant for detoxification and elimination of environmental toxins and free radicals, i.e., reactive oxygen species (ROS) that cause damage to cellular functions by oxidizing lipids, proteins and DNA. In addition to serving as an antioxidant, GSH plays an important role in cell differentiation, proliferation and apoptosis [11, 13–15]. There is also ample evidence on the role of glutathione in both innate and adaptive immune functions and on its anti-inflammatory role [13, 16–18].

Some studies provide evidence of the prenatal and perinatal onset for developmental abnormalities that lead to autism [19–21]. Children are more vulnerable than adults to oxidative stress, because of their low GSH levels [22, 23]. The risk from deficits in detoxification capacity in infants is higher because some environmental factors that induce oxidative stress accumulate in the placenta, and are found at higher concentrations in developing infants than in their mothers.

The biological activity of GSH resides in the sulfhydryl (thiol) group (SH) of cysteine. It acts as a reducing agent, and protects the cells from the deleterious effects of ROS by neutralizing them. In this process, GSH is oxidized to GSSG by glutathione peroxidase (GPx). GSSG can be recycled back to GSH by NADPH-dependent glutathione reductase (GR). In healthy cells and tissues, most of the total glutathione (tGSH) pool is in the GSH form, and less than 1 % [24] or 1.2 % [25] exists in the GSSG form. GSH and GSSG are the primary determinants of redox status in all human cells. A decrease in GSH-to-GSSG redox ratio is a marker of oxidative stress.

Extensive evidence from our and other groups suggests a role of oxidative stress in the development and clinical manifestation of autism. The levels of oxidative stress markers for lipid peroxidation, protein oxidation and/or DNA oxidation are increased in the blood [3, 5, 26–28], urine [29] and brains [3, 30–34] of autistic subjects as compared with control subjects. In addition, the activities of antioxidant enzymes and the levels of antioxidant proteins, namely transferrin (iron-binding protein) and ceruloplasmin (copper-binding protein) are decreased in the blood samples from autistic subjects [26–28, 35]. Several clinical studies have reported lower GSH levels and GSH/GSSG ratio in the plasma of individuals with autism [36–40]. However, the status of antioxidant capacity in the brains of individuals with autism has not been studied previously.

Brain tissue is highly heterogeneous, with specific functions localized in specific areas of the brain. The majority of free radicals, i.e., ROS, are produced in the mitochondria during oxidative metabolism and energy production, and the electron transport chain (ETC) in mitochondria is a prime source of ROS generation [41, 42]. We recently reported brain region–specific deficits in expression levels of mitochondrial ETC complexes in the cerebellum and the frontal and temporal cortices of children with autism [30]. Interestingly, the levels of ETC complexes were unaffected in the parietal and occipital cortices in autistic subjects compared to control subjects. In addition, increased lipid peroxidation was observed in the cerebellum and temporal cortex of autistic subjects, but not in other brain regions [30]. In view of the brain region–specific oxidative damage and mitochondrial ETC defects in autism, it was of interest to examine glutathione redox status in different brain regions (cerebellum and frontal, temporal, occipital and parietal cortices) from autism and age-matched control subjects.

Materials and Methods

Autism and Control Subjects

Samples of postmortem frozen brain regions, i.e., the cerebellum, and the cortices from the frontal, temporal, parietal and occipital lobes from autistic ($N = 7–10$ for different brain regions) and age-matched, typically developed, control subjects ($N = 9–10$) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The age (mean $\pm$ SE) for autistic subjects was 12.6 ± 3.2 years, and for control subjects, 12.4 ± 3.3 years. All brain samples were stored at -70 °C. This study was approved by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities.

The case histories for the autistic and control subjects are summarized in Table 1. Donors with autism had met the diagnostic criteria of the Diagnostic and Statistical Manual-IV (DSM-IV) for autism. The Autism Diagnostic Interview-Revised (ADI-R) test was performed for donor UMB #s 4671, 4849, 1174, 797, 1182, 4899 and 1638. Each donor's impairments in social interaction, qualitative

Table 1 Case history of autism and control donors of brain tissue samples

Brain tissue (UMB #)	Diagnosis	Autism Diagnostic tests	Age (year)	Sex	PMI (h)	Medications	Cause of death
4671	Autism	ADIR, VABS, BSID-II	4.5	F	13		Multiple injuries from fall
1349	Autism	ADOS, VABS, BSID-II	5.6	M	39		Drowning
4849	Autism	ADIR, BSID-II, CARS	7.5	M	20		Drowning
1174	Autism	ADIR, VABS	7.8	F	14	Depakote, Tegretol	Multiple-system organ failure
4231	Autism		8.8	M	12	Zyprexia, Reminyl	Drowning
797	Autism	ADIR	9.3	M	13	Desipramine	Drowning
1182	Autism	ADIR	10.0	F	24		Smoke inhalation
4899	Autism	ADIR	14.3	M	9	Trileptal, Zoloft, Clonidine, Melatonin	Drowning
1638	Autism	ADIR	20.8	F	50	Zoloft, Zyprexa, Mellaril, Depoprovera	Seizure-related
5027	Autism	WISC-R, Bender-Gestalt	38.0	M	26	Respirdal, Luvox	Obstruction of bowel
4670	Control		4.6	M	17		Commotio Cordis from an accident
1185	Control		4.7	M	17		Drowning
1500	Control		6.9	M	18		Motor vehicle accident
4898	Control		7.7	M	12	Concerta, Clonidone	Drowning
1708	Control		8.1	F	20		Motor vehicle accident
1706	Control		8.6	F	20		Rejection of cardiac allograft transplantation
1407	Control		9.1	F	20	Albuterol, Zirtec, Alegra, Rodact, Flovent, Flonase	Asthma
4722	Control		14.5	M	16		Motor vehicle accident
1846	Control		20.6	F	9		Motor vehicle accident
4645	Control		39.2	M	12		Arteriosclerotic heart disease

ADI-R Autism Diagnostic Interview Revised, *ADOS* Autism Diagnostic Observation Scale, *VABS* Vineland Adaptive Behavioral Scale, *BSID-II* Bayley Scales of Infant Development-Second Edition, *CARS* Childhood Autism Rating Scale, *WISC-R* Wechsler Intelligence Scale for Children-Revised

abnormalities in communication, and restricted, repetitive and stereotyped patterns of behavior were consistent with the diagnosis of autism, according to the results of the ADI-R diagnostic algorithm. All donors with autism exceeded the cut-off score in these parameters. The diagnosis of autism was assigned to donor UMB # 1349 after extensive evaluation of behavioral tests, including the Autism Diagnostic Observation Schedule (ADOS), Vineland Adaptive Behavioral Scale (VABS), and Bayley Scales for Infant Development-II (BSID-II). In addition to the ADI-R, UMB # 4849 was also evaluated by the BSID-II and the Childhood Autism Rating Scale (CARS), which indicated moderate to severe autism, and autism in UMB # 4671 was also verified by the VABS and BSID-II. Regressive autism, in which early development is normal but it is followed by loss of previously acquired language and/or social skills, was suggested in five autism cases (UMBs # 1349, 4849, 1182, 4899, 1638).

Preparation of Homogenates

The coded brain tissue samples (50–60 mg each) from autistic and control subjects were homogenized using a Polytron Tissue Trearor homogenizer with a 7.0-mm diameter stainless steel probe. The extraction solution consisted of formic acid (0.1 % v/v), potassium chloride (1.2 % w/v), EDTA (1 mM), bathophenanthroline disulfonic acid (2.4 mM) in serine-borate buffer (50 mM Tris–HCl, 25 mM borate, 25 mM serine and 100 μM diethylene-triamine pentaacetic acid; pH 7.0). The volume of the extraction solution was 750 μl (pH 2.8). The homogenization was performed twice for 30 s per sample at 4 °C,

followed by centrifugation at 18,000×*g* for 10 min at 4 °C. The supernatants were processed for assaying GSH and GSSG as described below.

Assay of GSH and GSSG

GSH and GSSG in brain tissues were measured using a modification of a method described by Santori et al. [43]. 100 µl of 10 mM iodoacetic acid in 10 mM aqueous ammonium bicarbonate and 0.5 % ammonia (V/V), pH 9.5 was added to 100 µl of above brain extracts or standards (GSH, GSSG). An aliquot of 50 ng of the internal standard (glutathione ethyl ester, i.e. GSHee) was added to each solution. The mixture was incubated in the dark for 1 h at 20 °C. Acetonitrile (400 µl) was added to stop the reaction and to precipitate the proteins. The samples were centrifuged, and the GSH and GSSG in the supernatants were separated by high performance liquid chromatography (HPLC) and measured by mass spectrometry (MS), following the method of Loughlin et al. [44]. The GSH and GSSG were detected in SRM (selected reaction monitoring) mode with a triple quadruple MS (Sciex API 3000; Ontario, Canada). The range of quantification for GSH was 150–150,000 nM and that of GSSG was 50.5–50,500 nM. In each sample, total glutathione (tGSH) level was calculated as [GSH + 2GSSG], and % GSSG was calculated as [(GSSG/tGSSG) × 100]. After the study was completed, the samples were decoded, and the contents of GSH, GSSG and tGSH, the redox ratio of GSH to GSSG, and % GSSG of tGSH were compared in the autism and control groups by unpaired student's *t* test.

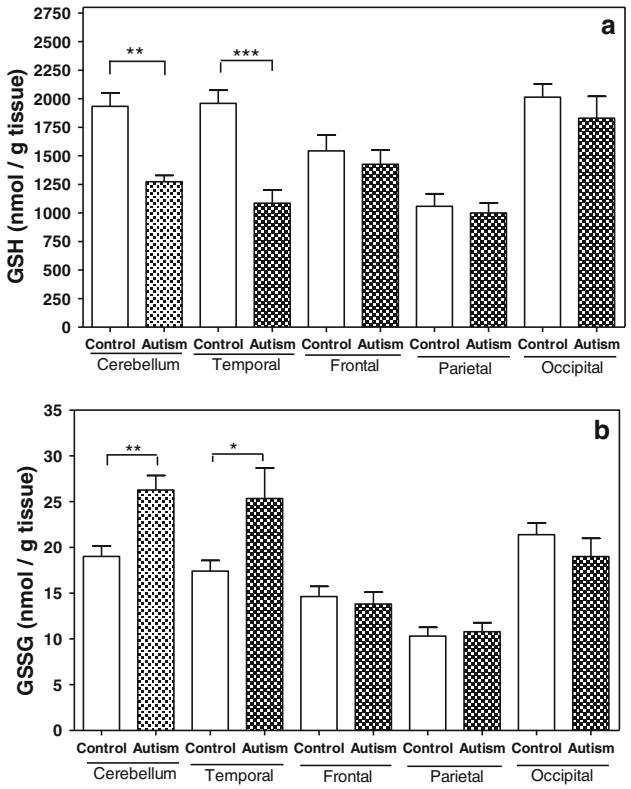

Fig. 1 Levels of reduced form of glutathione (GSH) and oxidized form of glutathione (GSSG) in the cerebellum and different regions of the cerebral cortex in subjects with autism and age-matched control subjects. There was a significant decrease in GSH levels (**a**) and increase in GSSG levels (**b**) in the cerebellum and temporal cortex in autism compared with the control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). No significant change in the levels of GSH and GSSG was observed in the frontal, parietal and occipital cortices between the autism and control groups

Results

The levels of GSH and GSSG in the brain tissue samples from the cerebellum and frontal, temporal, parietal and occipital cortices from individuals with autism and age-matched normal subjects are represented in Fig. 1a, b, respectively. The levels of GSH (Fig. 1a) were significantly decreased by 34.2 % in the cerebellum ($p = 0.001$), and by 44.6 % in the temporal cortex ($p = 0.0008$) in autistic subjects compared to control subjects. There was also a significant increase in the levels of GSSG (Fig. 1b) by 38.2 % in the cerebellum ($p = 0.0021$) and by 45.5 % in the temporal cortex ($p = 0.0214$) in autistic subjects compared with the control group. On the other hand, the levels of GSH and GSSG were similar in other brain regions, i.e., frontal, parietal and occipital cortices between the autism and control groups (Fig. 1a, b).

Table 2 represents the data for tGSH levels, GSH/GSSG redox ratio, and % GSSG of tGSH in the cerebellum and different regions of cerebral cortex from autism and control subjects.

The comparison of the tGSH contents showed a significant decrease of tGSH levels by 32.9 % ($p = 0.0013$) in the cerebellum, and by 43.1 % ($p = 0.0011$) in the temporal cortex of subjects with autism as compared to control subjects (Table 2). In the control group, percent GSSG of tGSH was 0.97 and 0.91 in the cerebellum and temporal cortex respectively (Table 2), which is in agreement with the literature values of GSSG to be less than 1–1.2 % in the human tissues under normal conditions [24, 25]. In comparison to the control group, GSSG % in the autism group increased by twofold to 1.98 in the cerebellum ($p < 0.0001$), and by 2.4-fold to 2.19-fold in the temporal cortex ($p < 0.0001$) (Table 2), suggesting oxidative stress condition in autism. The redox ratio of GSH/GSSG, an indicator of oxidative stress was significantly reduced by 52.8 % in the cerebellum ($p < 0.0001$) and by 60.8 % in

Table 2 Redox ratio of GSH/GSSG, levels of total glutathione, and percentage of oxidized glutathione in the cerebellum and different regions of cerebral cortex in the autism and control groups

Basin tissue	GSH/GSSG redox ratio	Total glutathione (tGSH)	% GSSG of tGSH
Cerebellum			
Autism (A)	48.7 ± 1.7	1,326 ± 59	1.98 ± 0.07
Control (C)	103.4 ± 5.9	1,976 ± 119	0.97 ± 0.05
Change (A vs. C)	↓ 52.8 %	↓ 32.9 %	↑ 2.0-fold
p value	<0.0001	0.0013	<0.0001
Temporal cortex			
Autism (A)	44.7 ± 3.1	1,136 ± 120	2.19 ± 0.12
Control (C)	113.9 ± 8.2	1,996 ± 157	0.91 ± 0.07
Change (A vs. C)	↓ 60.8 %	↓ 43.1 %	↑ 2.4-fold
p value	<0.0001	0.0011	<0.0001
Frontal cortex			
Autism (A)	103.8 ± 4.3	1,453 ± 128	0.96 ± 0.04
Control (C)	105.7 ± 4.6	1,574 ± 140	0.94 ± 0.04
Change (A vs. C)	↓ 8 %	↓ 7.7 %	None
p value	ns	ns	ns
Parietal cortex			
Autism (A)	93.0 ± 3.0	1,022 ± 90	1.06 ± 0.03
Control (C)	103.0 ± 5.4	1,080 ± 116	0.98 ± 0.05
Change (A vs. C)	↓ 9.7 %	↓ 5.4 %	None
p value	ns	ns	ns
Occipital cortex			
Autism (A)	96.5 ± 2.9	1,868 ± 195	1.02 ± 0.03
Control (C)	94.4 ± 2.5	2,057 ± 118	1.04 ± 0.03
Change (A vs. C)	↑ 2.2 %	↓ 9.2 %	None
p value	ns	ns	ns

GSH reduced glutathione, *GSSG* oxidized glutathione. Total glutathione (tGSH) was calculated as [GSH + 2GSSG], and % GSSG of tGSH was calculated as [(GSSG/tGSH) × 100]. A significant decrease in GSH/GSSG redox ratio and tGSH levels, and increase in % GSSG of tGSH was observed in the cerebellum and temporal cortex in the autism group as compared with the control group. There was no significant change in these parameters in other brain regions, i.e. frontal, parietal, and occipital cortices between the autism and control groups

the temporal cortex ($p < 0.0001$) in autistic subjects compared with control subjects (Table 2). However, there was no significant change in the tGSH levels, GSH/GSSG redox ratio, and % GSSG of tGSH in other brain regions, i.e., frontal, parietal and occipital cortices between the autism and control groups (Table 2). Taken together, a decrease in GSH levels, increase in GSSG levels and % GSSG of tGSH, and a decrease in the redox ratio of GSH/GSSG in the cerebellum and temporal cortex from autism subjects, but not in other brain regions, suggest brain region-specific glutathione redox imbalance in autism.

There was no significant difference in postmortem interval (PMI) between the autistic and control groups. The mean ± SE of PMI was: 22.0 ± 4.2 h in the autism group (n = 10), and 16.1 ± 1.22 h in the control group (n = 10). Because GSH and GSSG levels were affected in the cerebellum and temporal cortex but not in the frontal, parietal and occipital cortices of individuals with autism, these findings also suggest that PMI was not a contributing factor to the alterations in GSH and GSSG levels observed in the cerebellum and temporal cortex of individuals with autism.

Discussion

ASDs are considered multi-factorial disorders in which environmental factors may act as a trigger in genetically susceptible individuals, and oxidative stress may serve as a common link between genes and environmental factors. GSH is a major intracellular antioxidant and plays a crucial role in the maintenance and regulation of the thiol-redox status of the cell. In its reduced form, GSH protects the proteins, lipids and DNA from free radicals–mediated damage by providing the reduced environment, and during this process, it gets oxidized to GSSG by GPx. Therefore, decreased levels of GSH and increased levels of GSSG are suggestive of the oxidative stress environment in cells and tissues. The redox ratio of the GSH/GSSG serves as an important indicator of redox environment in the cell and plays an important role in cell differentiation, proliferation and apoptosis [11, 13–15]. Several reports have suggested that decrease in GSH levels can also be associated with immune system dysfunction and inflammation [13, 16–18].

This is the first study to compare glutathione redox status in the brain regions of autistic subjects and

age-matched control subjects. Our results indicate that (a) the levels of GSH, tGSH and also the redox ratio of GSH to GSSG are significantly decreased, and GSSG content and % GSSG of tGSH are significantly increased in the cerebellum and temporal cortex of the brains of individuals with autism compared with age-matched control subjects, and (b) glutathione redox imbalance and oxidative stress in autism is brain region–specific because in the frontal, parietal and occipital cortices, GSH, GSSG, tGSH and GSH/GSSG were similar in the autism and control groups. Reduced glutathione-mediated redox status has also been previously reported in blood samples from individuals with autism [36–40]. In addition, several studies have provided evidence for GSH depletion and disturbances in glutathione homeostasis in other neurobehavioral and neurodegenerative disorders, including schizophrenia [45, 46], bipolar disorder [47], Parkinson's disease and Alzheimer's disease [18, 48].

Extensive evidence from our and other groups has indicated that oxidative stress and inflammatory markers are increased in autism [3, 5, 7–9]. Numerous clinical studies in autism have provided evidence for increased oxidative stress, as revealed by elevated lipid peroxidation [5, 26–28] and reduced antioxidant defense [26–28, 35]. Recent postmortem studies have also shown evidence of increased lipid, protein and DNA oxidation in the cerebellum and temporal cortex of individuals with autism compared with control subjects [3, 30–34]. However, oxidative stress condition may not be the sole mechanism responsible for the deficit in GSH content in the cerebellum and temporal cortex from subjects with autism. There are several pathways by which cells maintain intracellular GSH homeostasis, including GSH redox cycling, direct uptake, and de novo synthesis. Further studies are needed to understand whether synthesis, consumption and/or regeneration of GSH are affected in the brain of subjects with autism. GSH serves as an essential cofactor or substrate for GPx, glutathione S transferase, and glyoxalase I, which are involved in antioxidant defense or detoxification [49]. Recently, reduced levels of NADPH were reported in the plasma of children with autism compared to those of controls [39], which may affect NADPH-dependent GR activity and thus, recycling of GSSG to GSH.

The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria, and the ETC in mitochondria is a prime source for ROS generation [41, 42]. Accumulating clinical, genetic and biochemical evidence suggests that mitochondrial dysfunction in ASDs occurs more commonly than expected [50, 51]. Recently, we reported brain region–specific changes in the levels of ETC complexes in the cerebellum and the frontal and temporal cortices but not in the parietal and occipital cortices in children with autism [30]. Mitochondria contain approximately 10–15 % of GSH, which is synthesized in the cytosol and transported into the mitochondria via an energy-dependent transporter [52]. A decrease in GSH availability in the brains of individuals with autism suggests that mitochondria may also be subjected to altered redox status, which will promote mitochondrial damage via increased ROS and affect cellular energy production [53]. We have also reported that the activities of Ca^{2+}–Mg^{2+}-ATPase and Na^{+}–K^{+}-ATPase are affected in the cerebellum and the frontal cortex of autistic subjects [54].

Recent studies support a prenatal onset for developmental abnormalities leading to autism [19–21]. Several studies have reported the adverse effects of endogenous or xenobiotic-enhanced generation of ROS and the resultant oxidative stress on embryonic and fetal development [55]. GSH is the major endogenous antioxidant produced by the cells, which participates directly in the neutralization of ROS. Through direct conjugation, it detoxifies many xenobiotics and carcinogens. The depletion of GSH has been reported to enhance embryopathies [56]. Exposure of the developing embryo or fetus to radiation and xenobiotics, including drugs and environmental chemicals, can affect development by increasing ROS levels [56, 57]. Excess of ROS may alter development by oxidatively damaging cellular lipids, proteins and DNA, and/or by altering signal transduction via Ras, NFκB and related transducers [55].

GSH also plays a central role in cell death, including apoptotic cell death [13–15]. GSH depletion is a common feature and an early hallmark in apoptotic cell death in response to a variety of apoptotic stimuli [14, 15]. GSH levels have also been reported to affect caspase activity, transcription factor activation, Bcl-2 expression and function, thiol-redox signaling and phosphatidylserine externalization [13]. Several lines of evidence suggest the involvement of apoptosis in the cerebellum of autism subjects, including loss and atrophy of Purkinje cells [58–60], reduced levels of Bcl2 and increased levels of p53 [61]. We suggest that the alteration in brain glutathione homeostatasis observed in this study may also play a role in apoptotic cell death in the brains of individuals with autism.

Our results suggest that PMI cannot account for the observed brain region-specific glutathione redox imbalance in autism. Other factors, such as medications (reported for six autism cases, and two control cases), and regression (reported for five autism cases) do not seem to be contributing factors to the decrease in GSH levels and GSH/GSSG redox ratio in the cerebellum and temporal cortex in autism. However, further studies with a larger autistic group are needed to explore this issue.

The brain region-specific location of changes in GSH/GSSG observed in the cerebellum and temporal cortex

from autistic subjects in this study fits to the brain region specificity of other manifestations of autism. There is substantial evidence from neuroimaging and postmortem neuropathological studies that dysfunctions in the cerebellum and the temporal lobe may result in autistic symptoms. Loss of Purkinje and granule cells throughout the cerebellar hemispheres in autism has been reported [58–60]. Other studies suggested neuroimmune activation/neuroinflammation in the cerebellum [9] as well as the presence of autoantibodies against cerebellar proteins [62]. The neuropathological and immunological abnormalities have also been suggested in the temporal lobe of the brain in autism. The main autistic symptoms were seen most consistently with a neurological model involving bilateral dysfunction of the temporal lobes [63]. Positron emission tomography and voxel-based image analysis also showed localized dysfunction of the temporal lobes in children with autism [64]. Recent magnetic resonance imaging (MRI) studies have shown abnormalities in the superior temporal gyrus (STG) region of the brain in autism, which is of particular interest because of its role in language processing and social perception [65–67]. Gene expression profiles in this region provided evidence of increased transcript levels of many immune system–related genes and immune signaling pathways suggesting neuroimmune activation of the STG in autism [68]. Furthermore, fewer and smaller neurons in the fusiform gyrus (FG), located in the temporal lobe, have been reported in autism [69]. The functional MRI studies also showed hypoactivation of the FG in face perception tasks in autistic subjects [70, 71]. The changes observed in the glutathione levels in the cerebellum and temporal lobes of subjects with autism suggest that oxidative stress may be one of the contributing factors to these pathological changes in the cerebellum and temporal lobes.

In conclusion, this study implicates disturbance in glutathione homeostasis and deficit in glutathione antioxidant capacity in specific brain regions, i.e., cerebellum and temporal cortex, of individuals with autism. Our previous report on increased lipid peroxidation and deficit in mitochondrial ETC complexes in these brain regions of autistic subjects also suggests increased oxidative damage and mitochondrial dysfunction in autism. GSH deficit in many diseases has been linked to immune dysfunction, inflammation and apoptosis. Taken together, these studies indicate oxidative damage coupled with deficit in glutathione antioxidant status in the brain of autistic subjects that may be associated with mitochondrial dysfunction, inflammation and immune abnormalities in ASDs.

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Brain Region–Specific Decrease in the Activity and Expression of Protein Kinase A in the Frontal Cortex of Regressive Autism

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Abstract

Autism is a severe neurodevelopmental disorder that is characterized by impaired language, communication, and social skills. In regressive autism, affected children first show signs of normal social and language development but eventually lose these skills and develop autistic behavior. Protein kinases are essential in G-protein-coupled, receptor-mediated signal transduction and are involved in neuronal functions, gene expression, memory, and cell differentiation. We studied the activity and expression of protein kinase A (PKA), a cyclic AMP–dependent protein kinase, in postmortem brain tissue samples from the frontal, temporal, parietal, and occipital cortices, and the cerebellum of individuals with regressive autism; autistic subjects without a clinical history of regression; and age-matched developmentally normal control subjects. The activity of PKA and the expression of PKA (C-α), a catalytic subunit of PKA, were significantly decreased in the frontal cortex of individuals with regressive autism compared to control subjects and individuals with non-regressive autism. Such changes were not observed in the cerebellum, or the cortices from the temporal, parietal, and occipital regions of the brain in subjects with regressive autism. In addition, there was no significant difference in PKA activity or expression of PKA (C-α) between non-regressive autism and control groups. These results suggest that regression in autism may be associated, in part, with decreased PKA-mediated phosphorylation of proteins and abnormalities in cellular signaling.

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Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairment in social interactions and verbal/non-verbal communication skills, and restricted, repetitive and stereotyped patterns of behavior [1]. According to a recent report from the Centers for Disease Control and Prevention, the prevalence of ASDs is 1 in 110 for children 8 years of age [2]. The symptoms of ASDs are typically present before the age of 3 years, and are often accompanied by abnormalities in cognitive functioning, learning, attention, and sensory processing. While the causes of ASDs remain elusive, ASDs are considered to be heterogeneous and multifactorial disorders that are influenced by both genetic and environmental factors. The onset of autism is gradual in many children. However, in regressive autism, children first show signs of normal social and language development but lose these developmental skills at 15–24 months and develop autistic behavior [3]. The reported incidence of regressive autism varies in different studies from 15% to 62% of cases [4–7]. In a few cases, regression may significantly affect language, with lesser impact in other domains such as social interaction or imaginative play [4,8]. On the other hand, some children may regress especially in social functions and not in language [9].

Protein kinases are known to play important roles in cellular signaling pathways and are involved in brain development [10–13]. Protein kinase A (PKA) is a cyclic adenosine monophosphate (cAMP)–dependent protein kinase that is involved in cognitive functions and memory formation [14–18]. PKA consists of regulatory (R) and catalytic (C) subunits. Three genes encode for catalytic units (Cα, Cβ, and Cγ), and four other genes encode for regulatory units (RIα, RIβ, RIIα, and RIIβ) of PKA. PKA remains catalytically inactive when the levels of cAMP are low. The concentration of cAMP rises upon activation of adenylate cyclase by G protein-coupled receptors, and/or inhibition of cyclic nucleotide phosphodiesterase (PDE) enzyme. Under these conditions, cAMP binds to two binding sites on the regulatory subunits of PKA, which results in the release of the catalytic subunits. These free catalytic units of PKA can then phosphorylate proteins by transferring a phosphate group from ATP. Several studies have implicated the role of PKA in neuropsychiatric disorders such as schizophrenia, bipolar affective disorder, obsessive compulsive disorder, and panic disorders [19–22]. To date, no studies of PKA have been done in autism.

The intracellular levels of cAMP are controlled by PDE, which degrades the phosphodiester bond in cAMP. PDE regulates the localization, duration, and amplitude of cAMP signaling within subcellular domains. Multiple forms of PDEs have been identified

on the basis of substrate specificity. PDE4, 7, and 8 act on cAMP; PDE5, 6, and 9 act on cyclic guanosine monophosphate (cGMP); whereas PDE1, 2, 3, 10, and 11 act on both cAMP and cGMP. Recent evidence has suggested altered levels of PDE4 in the brains of individuals with autism [23].

Because the levels of PDE4 are altered in autism, and PKA is involved in neuropsychiatric disorders, it was of interest to compare the activity and protein levels of PKA in different brain regions in autism (regressive and non-regressive) and age-matched control subjects. Our study suggests that PKA activity and expression are decreased in the frontal cortex of individuals with regressive autism as compared with control subjects. Such changes were not observed in individuals with non-regressive autism.

Materials and Methods

Autism and Control Subjects

Samples of postmortem frozen brain regions, i.e., the cerebellum, and the cortices from the frontal, temporal, parietal, and occipital lobes from autistic (N = 7–10 for different brain regions) and age-matched, typically developed, control subjects (N = 9–10) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The age (mean ± S.E.) for autistic subjects was 12.6±3.2 years, and for control subjects, 12.4±3.3 years. All brain samples were stored at −70°C.

The case history and clinical characteristics for the autism and control subjects are summarized in Table 1. Donors with autism had met the diagnostic criteria of the Diagnostic and Statistical Manual-IV for autism. The Autism Diagnostic Interview-Revised (ADI-R) test was performed for the donors UMB #s 4671, 4849, 1174, 797, 1182, 4899, and 1638 (Table 2). Each donor's impairments in social interaction, qualitative abnormalities in communication, and restricted, repetitive and stereotyped patterns of behavior are consistent with the diagnosis of autism, according to the results of the ADI-R diagnostic algorithm. All donors with autism exceeded the cut-off score in these parameters. The diagnosis of autism was assigned to donor UMB # 1349 after extensive evaluation of behavioral tests, including the Autism Diagnostic Observation Schedule (ADOS), Vineland Adaptive Behavioral Scale (VABS), and Bayley Scales for Infant Development-II (BSID-II). In addition to the ADI-R, UMB # 4849 was also evaluated by the BSID-II and Childhood Autism Rating Scale (CARS), which indicated moderate to severe autism, and autism in UMB # 4671 was also verified by the VABS and BSID-II. Table 3 shows scores for the VABS test, which assesses adaptive behavior in four domains: communication, daily living skills, socialization, and motor skills.

In this study, the subjects with autism were divided into two subgroups: regressive autism and non-regressive autism, depending on the pattern of onset of symptoms for autism. Regressive autism is a type of autism in which early development is normal, followed by a loss of previously acquired skills. Language is the most common area that regresses; this regression can be accompanied by more global regression involving loss of social skills and social interest. On the other hand, in non-regressive autism, the child never gains normal language and social skills, and initial symptoms are delayed speech development, and/or delay in development of social skills and in nonverbal communication. These children do not demonstrate regression in terms of loss of language or social skills.

Ethics statement. This study was approved by the Institutional Review Board (IRB) of the New York State Institute for Basic Research in Developmental Disabilities. The IRB reviewed this study in accordance with New York State Regulations and the HHS Office for Human Research Protections, including the "Human Subject Decision Chart 1," and found that *the research does not involve human subjects* because "the research does not involve intervention or interaction with the individuals", nor "is the information individually identifiable". The subjects cannot be identified, directly or through identifiers linked to the system, and the consent is not required.

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 β-mercaptoethanol, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at 4°C. For extraction of protein kinases, the homogenates were mixed with an equal volume of extraction buffer containing 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM Na_3VO_4, 100 mM NaF, and 2 μg/ml leupeptin. The samples were allowed to stand on ice for 10 min, and then centrifuged at 135,000 g for 20 min at 4°C. The supernatants were collected, and the concentrations of total proteins in the supernatants were measured by the biocinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL).

Assay for PKA Activity

PKA activity was measured using the solid phase enzyme-linked immunosorbent assay (ELISA) kit from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). In this assay, the substrate of PKA was pre-coated on the wells of a microplate. The microplate wells were soaked with 50 μl of kinase assay dilution buffer for 10 min. The buffer was then carefully aspirated from each well, and the brain samples were added to the appropriate wells. The kinase reaction was initiated by adding 10 μl ATP, and was carried out for 90 min at 30°C. It was terminated by emptying the contents of each well. A phosphosubstrate–specific antibody was added to the wells except in blank, and incubated for 60 min at room temperature, followed by washing 4 times with wash buffer. The peroxidase-conjugated secondary antibody was then added except in blank, and incubation was done for 60 min at room temperature. The wells were again washed 4 times with wash buffer. The color was developed with tetramethylbenzidine substrate, and it was proportional to the phosphotransferase activity of PKA. The reaction was stopped with acid-stop solution, and the absorbance was measured at 450 nm in a microplate reader. The absorbance was divided by the concentration of total protein (μg) in each sample, and the data are represented as relative PKA activity.

Western Blot Analysis

Total protein (15 μg) from brain homogenates of subjects with regressive- and non-regressive autism or control subjects was separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline containing 5% fat-free dried milk for 2 h at room temperature, and further incubated overnight at 4°C with polyclonal antibody against C-subunit (isoform α) of PKA (Cell Signaling Technology Inc., Danvers, MA). The membrane was then washed 3 times with TBS-0.05% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The membrane was washed again, and the immunoreactive protein was visualized using enhanced chemiluminescent reagent.

Table 1. Case history and clinical characteristics of autism and control donors of brain tissue samples.

Brain tissue (UMB #)	Diagnosis	Autism Diagnostic tests	Age (y)	Sex	PMI (h)	Regressive autism	Other medical conditions	Medications	Cause of death
4671	Autism	ADIR, VABS, BSID-II	4.5	F	13	No			Multiple injuries from fall
1349	Autism	ADOS, VABS, BSID-II	5.6	M	39	Yes			Drowning
4849	Autism	ADIR, BSID-II, CARS	7.5	M	20	Yes	Lead poisoning		Drowning
1174	Autism	ADIR, VABS	7.8	F	14	No	Seizures	Depakote, Tegretol	Multiple-system organ failure
4231	Autism		8.8	M	12	No	Hyperactivity	Zyprexia, Reminyl	Drowning
797	Autism	ADIR	9.3	M	13	No	Attention deficit disorder, migraine headache	Desipramine	Drowning
1182	Autism	ADIR	10.0	F	24	Yes			Smoke inhalation
4899	Autism	ADIR	14.3	M	9	Yes	Seizures	Trileptal, Zoloft,Clonidine, Melatonin	Drowning
1638	Autism	ADIR	20.8	F	50	Yes	Seizures, Attention deficit hyperactivity disorder	Zoloft, Zyprexa, Mellaril, Depoprovera	Seizure-related
5027	Autism	WISC-R, Bender-Gestalt	38.0	M	26	No		Respirdal, Luvox	Obstruction of bowel
4670	Control		4.6	M	17				Commotio Cordis from an accident
1185	Control		4.7	M	17				Drowning
1500	Control		6.9	M	18				Motor vehicle accident
4898	Control		7.7	M	12		Hyperactive disorder	Concerta, Clonidone	Drowning
1708	Control		8.1	F	20				Motor vehicle accident
1706	Control		8.6	F	20		Congenital heart disease with heart transplant		Rejection of cardiac allograft transplantation
1407	Control		9.1	F	20		Asthma allergies	Albuterol, Zirtec, Alegra, Rodact, Flovent, Flonase	Asthma
4722	Control		14.5	M	16				Motor vehicle accident
1846	Control		20.6	F	9				Motor vehicle accident
4645	Control		39.2	M	12				Arteriosclerotic heart disease

ADI-R: Autism Diagnostic Interview Revised.
ADOS: Autism Diagnostic Observation Scale.
VABS: Vineland Adaptive Behavioral Scale.
BSID-II: Bayley Scales of Infant Development-Second Edition.
CARS: Childhood Autism Rating Scale.
WISC-R: Wechsler Intelligence Scale for Children-Revised.
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Table 2. Autism Diagnostic Interview-Revised test scores in donors of brain tissue samples.

Autism Diagnostic Interview-Revised (ADI-R)[a]							
Diagnostic Algorithm	**Cutoff score for autism**	**UMB 4671**	**UMB 4849**	**UMB 1174**	**UMB 797**	**UMB 4899**	**UMB 1638**
Impairments in reciprocal social interaction (Scores:0–30)	10	26	22	22	24	22	21
Abnormalities in communication:							
Verbal (Scores:0–26)	8	-	18	-	20	-	-
Non-verbal (Scores: 0–14)	7	13	N/A	11	13	14	11
Restricted, repeated and stereotyped behavior (Scores: 0–12)	3	3	8	6	6	8	7
Abnormalities of development evident at or before 36 months	1	5	3	5	-	4	5

a: Higher score represents greater impairment.
UMB 1182: ADI-R was conducted but the scores are not available. The donor met the criteria for a diagnosis of autism.
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Because PKA (C-α) and β-actin have similar molecular weights (42 KDa), polyclonal antibody against PKA (C-α) was stripped from nitrocellulose membrane, and the membrane was re-probed with monoclonal antibody against β-actin (loading control). The densities of all protein bands were measured by NIH Image J software, and the density of PKA (C-α) band was normalized with the density of β-actin for each sample.

Statistical Analysis

Initially, autistic and control cases were collected as age-matched pairs. As data for both members of a pair were not available in all cases, and data were approximately normally distributed, unpaired two-tailed t-tests were employed to make comparisons of PKA activity in various brain regions, and of overall PKA density between autistic vs. control cases. Comparisons among controls and autistic cases showing or not showing clinical signs of regression in function were made using one-way analysis of variance (ANOVA). To guard against type I error, a Bonferroni adjustment for multiple comparisons was made to the t-tests of multiple brain regions, and for the pairwise *post-hoc* t-tests comparing each pair of the three groups that were compared in the overall ANOVA. For purposes of this adjustment, tests of different hypotheses, i.e., of activity levels and of protein contents of PKA, were not considered to be multiple comparisons.

Results

PKA Activity in Different Brain Regions of Individuals with Autism and Age-Matched Control Subjects: Relationship with Regression in Autism

The activity of PKA was measured in the brain homogenates from the frontal, temporal, occipital, and parietal cortices, and the cerebellum in autistic and control subjects (Fig. 1). When all autism cases (regressive and non-regressive) were compared with the age-matched control group, no significant difference was found in PKA activity in any of these brain regions, although PKA activity in the frontal cortex was found to be reduced by 34.7% in the autism vs. control group. When the autism group was divided into two sub-groups (regressive and non-regressive), depending on whether there was a clinical history of regression or not, unadjusted two-tailed t-test showed a significant decrease in PKA activity in the frontal cortex of individuals with regressive autism as compared to the developmentally normal control group ($p = 0.0278$) and the non-regressive autism group

Table 3. Vineland Adaptive Behavioral Scales diagnostic test for autism in donors of brain tissue samples.

Vineland Adaptive Behavioral Scales (VABS)[a]							
	UMB 1349				**UMB 4671**		**UMB 1174**
	At age: 25 months		**At age: 33 months**		**At age: 39 months**		**At age: 6.4 y**
Domain (Scores:20–160)	**Standard Score**	**Age equivalent performance**	**Standard Score**	**Age equivalent performance**	**Standard Score**	**Age equivalent performance**	**Standard score**
Communication	57	9 months	69	18 months	52	10 months	41
Daily living skills	65	16 months	62	16 months	54	14 months	22
Socialization	60	9 months	71	17 months	51	4 months	52
Motor skills	-	-	-	-	65	24 months	-
Composite	-	-	-	-	51	13 months	35

a: Higher score represents better function.
According to the medical histories for UMB-4231 and UMB-5027, the donors had psychological evaluation, and met the criteria for a diagnosis of autism. Detailed information is not available.
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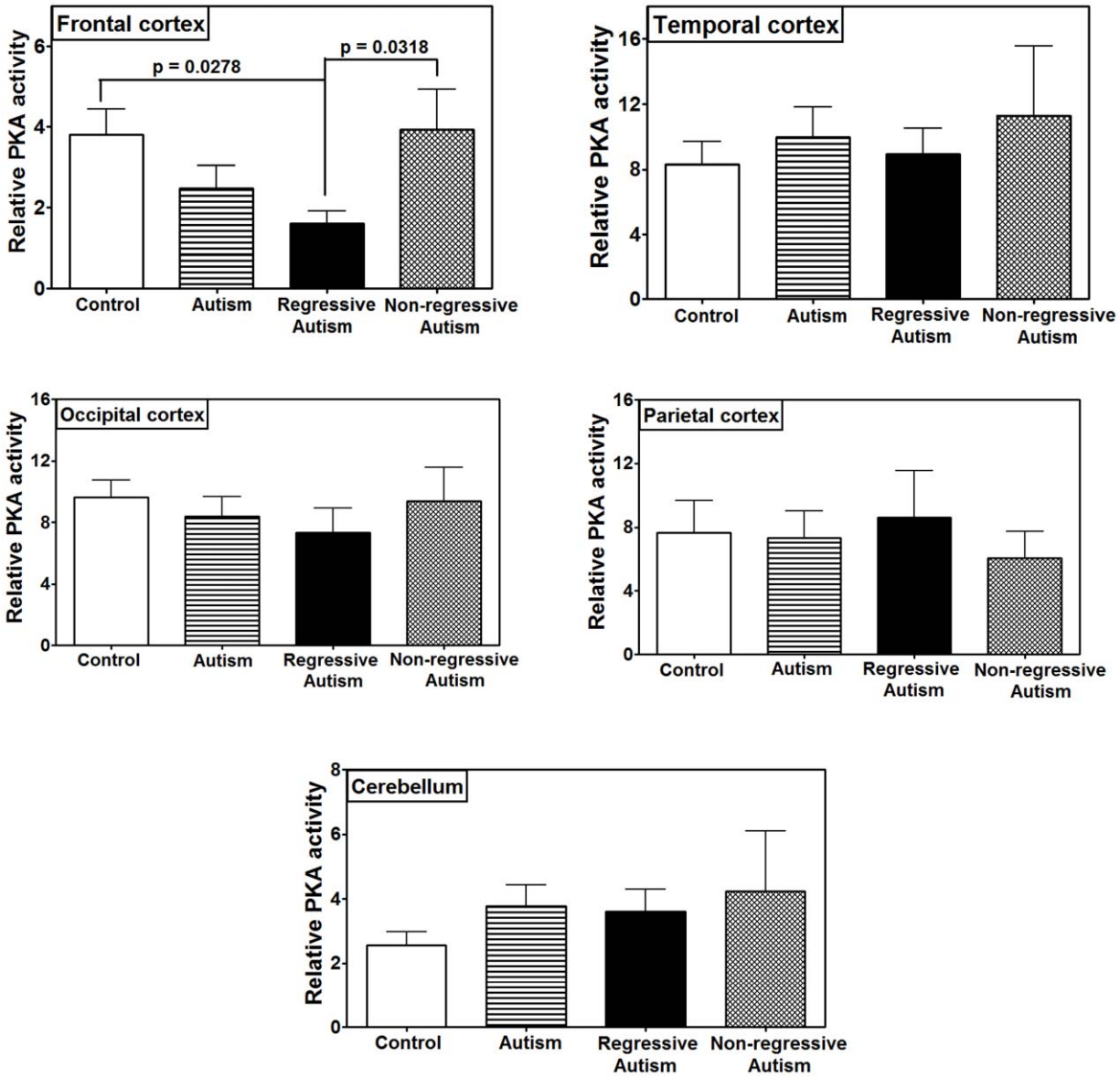

Figure 1. PKA activity in different brain regions from regressive autism, non-regressive autism, and age-matched control subjects. The autism group comprises combined regressive and non-regressive autism sub-groups. Brain homogenates were prepared, and activity of PKA was measured as described in Materials and Methods. Data represent mean ± S.E.
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($p = 0.0318$), but these differences did not remain significant after application of the adjustment for multiple comparisons. The mean ± S.E. of PKA activity in the frontal cortex was: 2.48 ± 0.57 in autism (regressive+non-regressive), 1.60 ± 0.31 in regressive autism, 3.94 ± 0.99 in non-regressive autism, and 3.80 ± 0.65 in control groups. The alteration in PKA activity was specific to the frontal cortex in regressive autism because it was not observed in other regions of the brain, i.e., the cerebellum and the temporal, parietal, and occipital cortices, suggesting that the changes observed in PKA activity were brain region–specific in regressive autism. PKA activity was also similar in all of the brain regions between non-regressive autism and control groups.

There was no significant difference in postmortem interval (PMI) between the autistic and control groups, or between the regressive autism and non-regressive autism groups. The mean ± S.E. of PMI was: 22.0 ± 4.2 in the autism groups (regressive+non-regressive, $n = 10$), 16.1 ± 1.22 in the control group ($n = 10$), 28.4 ± 7.2 in regressive autism ($n = 5$), and 15.6 ± 2.6 in the non-regressive autism group ($n = 5$). We also studied whether there was an inverse correlation between PMI and PKA activity. Correlation analysis between PMI and PKA activity for all autistic and control subjects did not reveal any such association (data not shown). Furthermore, the cerebellum and the temporal, parietal, and occipital cortices were not affected in subjects with regressive autism in comparison with control subjects, while the frontal cortex was affected in these individuals. These results suggest that PMI was not a contributing factor to the observed alteration in PKA activity in the frontal cortex of individuals with regressive autism. There was also no significant difference in age (mean ± S.E.) between the regressive autism (11.6 ± 2.7 years, $n = 5$) and non-regressive autism groups (13.7 ± 6.1 years, $n = 5$).

Protein Levels of Catalytic C-α Subunit of PKA in the Frontal Cortex of Individuals with Autism (Regressive and Non-Regressive) and Control Subjects

Because a decrease in PKA activity was observed in the frontal cortex of subjects with regressive autism as compared to control subjects and subjects with non-regressive autism, we analyzed whether the decreased activity of PKA is related to the reduced protein contents of PKA. The protein contents of the catalytic Cα unit of PKA were analyzed in the frontal cortex of individuals with autism (regressive and non-regressive) and age-matched controls by Western blotting (Fig. 2 A). The relative densities of the protein contents of PKA (C-α) normalized with β-actin are shown in Fig. 2 B. A one-way ANOVA comparing regressive and non-regressive autism cases and controls showed a significant difference in the protein contents among these three groups ($F_{[df=2,15]} = 9.770$, $p = 0.002$). *Post-hoc* pairwise comparisons among the groups revealed a significant decrease in the protein contents of PKA (C-α) in individuals with regressive autism (mean ± S.E = 0.34±0.09) as compared to control (mean ± S.E. = 0.64±0.05, $p = 0.019$, Bonferroni-adjusted) and individuals with non-regressive autism (mean ± S.E. = 0.83±0.09, $p = 0.002$, Bonferroni-adjusted), suggesting that the protein contents of PKA are affected in regressive autism. PKA contents were similar between non-regressive autism and control groups, and when the entire autism group (regressive and non-regressive) was compared with the control group.

Discussion

ASDs are complex neurodevelopmental disorders. The complexity of ASDs is further increased because some affected individuals fall in the sub-group of regressive autism [7]. Behavioral changes in regressive autism fall into two broad domains: (a) loss of vocalization and (b) loss of social skills. The rate of regressive autism varies from 15% to 62% of cases in different studies [4–7]. While Lord *et al.* reported that 29% of the children they studied who were diagnosed with autism had lost language skills for meaningful words, and another 9% lost non-word vocalizations [5], Goldberg *et al.* reported regression in 62% of children [4]. Loss of spoken words generally associates with loss of social behavior [6], but some affected children show only loss of social skills [4]. We report here that individuals with regressive autism have decreased PKA activity in the frontal cortex of the brain. This decreased PKA activity in autistic regression may be attributed to the decreased protein contents of PKA because the protein content of PKA (C-α subunit) was also decreased in the frontal cortex of individuals with regressive autism. Interestingly, such changes were not observed in other brain regions of individuals with regressive autism, or in the frontal cortex and other brain regions of individuals with non-regressive autism. These results suggest that alterations in PKA activity and PKA expression are specific to the frontal lobe in regressive autism.

Our results suggest that PMI and age cannot account for the observed alteration in PKA in regressive autism. Other factors, such as comorbidity with seizure disorder, reported for three of 10 autism cases (of which two had regressive autism, and one had non-regressive autism), and medications, reported for two regressive autism cases, four non-regressive autism cases, and two control cases, do not seem to be contributing factors to the altered activity or expression of PKA in regressive autism.

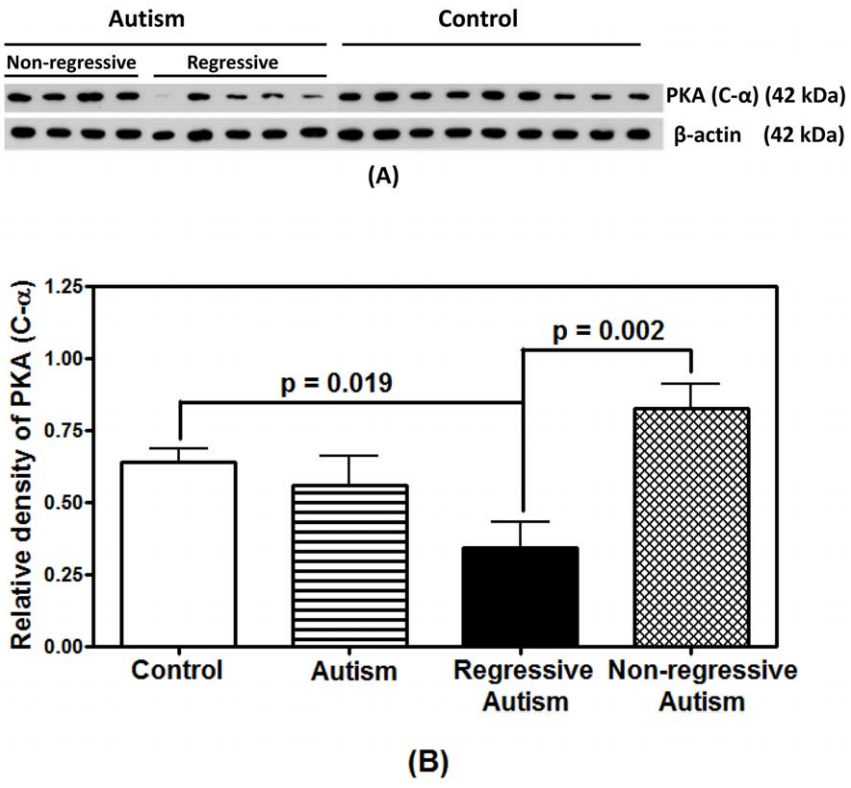

Figure 2. Relative protein levels of PKA (C-α) in the frontal cortex of regressive autism, non-regressive autism, and age-matched control subjects. Western blot analyses of C-α subunit of PKA in the frontal cortex of individuals with regressive and non-regressive autism, and age-matched control subjects are represented in Fig. 2A. The relative density of PKA (C-α) normalized with the density of β-actin (loading control) is shown in Fig. 2B. Data represent mean ± S.E.
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However, further studies with a larger autistic group should be done to explore this issue.

cAMP is one of the key factors for neuronal outgrowth, plasticity, and regeneration. Members of the cAMP-dependent second-messenger pathways participate in the regulation of cellular growth and differentiation and are also implicated in a variety of embryonic stages including brain development [24]. The PKA pathway is also recognized as an essential component in memory formation. Several studies in Drosophila have demonstrated the role of PKA in memory formation [25–29]. Mutations in the rutabaga gene, which encodes adenylate cyclase, caused significant defects in short-term memory [25]. Reduced expression or activity of DC0 (the gene encoding the catalytic subunit of PKA) caused deficits in learning, short-term memory, and middle-term memory [26–28]. Studies have also shown that pharmacological agents such as cAMP analogs and rolipram (an inhibitor of PDE), which are known to increase PKA activity, could improve memory [30,31].

G-protein–coupled adenylate cyclase converts ATP to cAMP, which in turn binds to regulatory subunits of PKA. Following this event, catalytic subunits of PKA are released, which are the activated forms of PKA. PKA then phosphorylates and alters the activity of enzymes and many target proteins such as ion channels, chromosomal proteins, and transcription factors. cAMP response-binding protein (CREB) is one of the targets of PKA-mediated phosphorylation. CREB, upon activation by PKA, binds to certain DNA sequences (cAMP response elements), thereby stimulating the transcription of downstream genes and the synthesis of proteins. The CREB transcription factor is also required for long-term memory formation [32–34]. It is possible that a decrease in the activity of PKA in regressive autism may result in reduced phosphorylation of CREB, and thus reduced transcription and altered synthesis of some proteins.

Given that PKA is activated by cAMP, and PDE regulates the levels of cAMP, a discussion on PDE becomes imperative. Altered levels of PDE4 in the cerebella of autism subjects were reported by Fatemi and group [23]. Other studies have suggested a role of PDE4 in learning and memory in behavioral models of mice, rats, and monkeys [35,36]. PDE4 is also reported to be involved in behavior sensitivity to antidepressant drugs in animals [37]. PDE inhibitors such as rolipram could improve object recognition [38,39], passive avoidance [40,41], radial arm maze [40–42], Morris water maze [43], and contexual fear conditioning [30,43,44]. PDE4 has also been studied as a potential therapeutic target for depressive disorders. It has been suggested that rolipram may have potential therapeutic benefits for major depression [45], Alzheimer's disease [36,46], Parkinson's disease [47,48], schizophrenia [49,50], and tardive dyskinesia [51,52].

Several reports suggest that some proteins related to the PKA pathway are involved in autism. Extensive evidence indicates hyperserotonemia in autism [53–55]. PKA regulates serotonergic activity in the brain [56]. Galter and Unsicker [57] reported that co-activation of cAMP- and tyrosine receptor kinase B (TrkB)–dependent signaling pathways plays an important role in maintaining the serotonergic neuronal phenotype. TrkB is also regulated by the cAMP/CREB pathway in neurons [58]. Furthermore, transcriptional activity of the engrailed-2 gene is also regulated by PKA [59]. The importance of engrailed can be envisioned because of its crucial roles in brain development [60] and in the development of autism [61–65].

In conclusion, this study suggests that the frontal cortex may be the region of the brain involved in regressive autism, where abnormalities such as decreased activity and expression of PKA can affect the signal transduction. It may have multiple effects on signal transduction pathways, which may also influence serotonergic neurons, TrkB, and engrailed-2, all of which have been suggested to be involved in the development of autism.

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Author Contributions

Conceived and designed the experiments: AC. Performed the experiments: LJ. Analyzed the data: AC VC MJF. Contributed reagents/materials/analysis tools: AC. Wrote the paper: AC VC.

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Research Paper

Reduced Activity of Protein Kinase C in the Frontal Cortex of Subjects with Regressive Autism: Relationship with Developmental Abnormalities

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Abstract

Autism is a neurodevelopmental disorder with unknown etiology. In some cases, typically developing children regress into clinical symptoms of autism, a condition known as regressive autism. Protein kinases are essential for G-protein-coupled receptor-mediated signal transduction, and are involved in neuronal functions, gene expression, memory, and cell differentiation. Recently, we reported decreased activity of protein kinase A (PKA) in the frontal cortex of subjects with regressive autism. In the present study, we analyzed the activity of protein kinase C (PKC) in the cerebellum and different regions of cerebral cortex from subjects with regressive autism, autistic subjects without clinical history of regression, and age-matched control subjects. In the frontal cortex of subjects with regressive autism, PKC activity was significantly decreased by 57.1% as compared to age-matched control subjects ($p = 0.0085$), and by 65.8% as compared to non-regressed autistic subjects ($p = 0.0048$). PKC activity was unaffected in the temporal, parietal and occipital cortices, and in the cerebellum in both autism groups, i.e., regressive and non-regressed autism as compared to control subjects. These results suggest brain region-specific alteration of PKC activity in the frontal cortex of subjects with regressive autism. Further studies showed a negative correlation between PKC activity and restrictive, repetitive and stereotyped pattern of behavior ($r = -0.084$, $p = 0.0363$) in autistic individuals, suggesting involvement of PKC in behavioral abnormalities in autism. These findings suggest that regression in autism may be attributed, in part, to alterations in G-protein-coupled receptor-mediated signal transduction involving PKA and PKC in the frontal cortex.

Key words: Autism; behavior; protein kinase C; protein kinases; regression; signal transduction.

INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairment in social interactions, verbal and non-verbal communication skills, and restricted, repetitive and stereotyped patterns of behavior [1]. Recently, Centers for Disease Control and Prevention reported the prevalence of ASDs to be 1 in 88 children in the United States [2]. The symptoms of ASDs usually start before the age of 3 years, and are often accompanied by abnormalities in cognitive functioning, learning, attention, and sensory processing. The cause of ASDs is not known. However, ASDs are considered as het-

erogeneous and multifactorial disorders that are influenced by genetic and environmental factors [3-5]. Several lines of evidence from our and other groups have suggested increased oxidative stress [3; 6-14], mitochondrial dysfunctions [8; 10; 15; 16], immune dysfunction and inflammation [10; 17-21] in autism.

The onset of autism is gradual in many children. However, in regressive autism, children first show sign of normal social and language development but lose these developmental skills at 15–24 months and develop autistic behavior [22]. The reported incidence of regressive autism varies from 15% to 62% of cases in different studies [23-26]. In few cases, regression may significantly affect language with lesser impact on other domains such as social interaction or imaginative play [23; 27]. On the other hand, some children may regress particularly in social functions and not in language [28].

The cause of regression in autism is not understood. Protein kinases are known to play important roles in cellular signaling pathways, and are involved in neurodevelopment [29-31]. The brain synapses are the building blocks of memory formation, and synaptic strength contributes to learning and memory [32]. The changes in neurotransmitters release, receptor sensitivity, and gene expression are involved in synaptic strength, structure and function. Because protein kinases-mediated phosphorylation modifies the functions of proteins, altered activities of protein kinases affect the synaptic efficacy.

Recently, we reported that the activity of cAMP-dependent protein kinase A (PKA) is decreased in the frontal cortex of subjects with regressive autism as compared to age-matched control subjects and autistic subjects without clinical history of regression [33]. Protein Kinase C (PKC), a ubiquitous phospholipid-dependent serine/threonine kinase, is another G-protein-coupled receptor-mediated kinase. PKC is known to be involved in signal transduction associated with the control of brain functions, such as ion channel regulation, receptor modulation, neurotransmitters release, synaptic potentiation/depression, and neuronal survival [34]. It also plays crucial roles in cell proliferation, differentiation and apoptosis. Neuronal tissues have high activity of PKC.

Genetic studies have suggested an involvement of PKC in autism [35; 36]. The analysis of genome-wide linkage and candidate gene association showed PKCβ gene (PRKCB1) linkage to a region on chromosome 16p in the neocotex of subjects with autism [35;36]. High-resolution single-nucleotide polymorphism genotyping and analysis of this region showed strong association of haplotypes in the PKCβ gene with autism. The present study was undertaken to compare the activity of PKC in the cerebellum and different regions of cerebral cortex from subjects with regressive and non-regressive autism and their age-matched control subjects. The relationship between PKC activity and behavioral abnormalities was also studied in autism.

MATERIALS AND METHODS

Autism and control subjects. Samples of postmortem frozen brain regions, i.e., the cerebellum, and cortices from the frontal, temporal, parietal and occipital lobes from autistic (N= 7-10 for different brain regions) and age-matched typically developed, control subjects (N= 9-10) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The age (mean ± S.E.) for autistic subjects was 12.6 ± 3.2 years, and for control subjects, 12.4 ± 3.3 years. All brain samples were stored at –70°C. This study was approved by the Institutional Review Board (IRB) of the New York State Institute for Basic Research in Developmental Disabilities.

Diagnostic classification. The case history and clinical characteristics for the autism and control subjects is summarized in Table 1. Donors with autism had met the diagnostic criteria of the Diagnostic and Statistical Manual-IV for autism. Autism Diagnostic Interview-Revised (ADI-R) test was performed for UMB # 4671, 4849, 1174, 797, 1182, 4899 and 1638 (Table 2). According to the results of ADI-R diagnostic algorithm, the donor's impairments in social interaction, qualitative abnormalities in communication and restricted, repetitive and stereotyped patterns of behavior are consistent with diagnosis of autism. All exceeded cut off score in each of these parameters. Diagnosis of autism was assigned to UMB # 1349 after extensive evaluation of behavioral tests, including Autism Diagnostic Observation Schedule (ADOS), Vineland Adaptive Behavioral Scale (VABS), and Bayley Scales for Infant Development-II, (BSID-II). In addition to ADIR, UMB # 4849 was also evaluated by BSID-II and Childhood Autism Rating Scale (CARS), which indicated moderate to severe autism, and autism in UMB # 4671 was also verified by VABS. Table 3 shows VABS test which assesses adaptive behavior in four domains: communication, daily living skills, socialization, and motor skills.

In this study, the subjects with autism were divided into two subgroups: regressive autism and non-regressive autism, depending on the pattern of onset of symptoms for autism. Regressive autism refers to a child where parents report an early history of

normal development, which is followed by a loss of previously acquired skills. Language regression is the most common form of regression but it can also be accompanied by more global regression involving loss of social skills and interest. On the other hand, in non-regressive autism, the child never gains normal language and social skills, and initial symptoms are delayed speech development, and/or delay in development of social skills and nonverbal communication. These children do not demonstrate regression in terms of loss of language or social skills.

Table 1. Case history and clinical characteristics of autism and control donors of brain tissue samples.

Brain tissue (UMB #)	Diagnosis	Autism Diagnostic tests	Age (y)	Sex	PMI (h)	Regressive autism	Other medical conditions	Medications	Cause of death
4671	Autism	ADIR, VABS, BSID-II	4.5	F	13	No			Multiple injuries from fall
1349	Autism	ADOS, VABS, BSID-II	5.6	M	39	Yes			Drowning
4849	Autism	ADIR, BSID-II, CARS	7.5	M	20	Yes	Lead poisoning		Drowning
1174	Autism	ADIR, VABS	7.8	F	14	No	Seizures	Depakote, Tegretol	Multiple-system organ failure
4231	Autism		8.8	M	12	No	Hyperactivity	Zyprexia, Reminyl	Drowning
797	Autism	ADIR	9.3	M	13	No	Attention deficit disorder, migraine headache	Desipramine	Drowning
1182	Autism	ADIR	10.0	F	24	Yes			Smoke inhalation
4899	Autism	ADIR	14.3	M	9	Yes	Seizures	Trileptal, Zoloft,Clonidine, Melatonin	Drowning
1638	Autism	ADIR	20.8	F	50	Yes	Seizures, Attention deficit hyperactivity disorder	Zoloft, Zyprexa, Mellaril, Depoprovera	Seizure-related
5027	Autism	WISC-R, Bender-Gestalt	38.0	M	26	No		Respirdal, Luvox	Obstruction of bowel
4670	Control		4.6	M	17				Commotio Cordis from an accident
1185	Control		4.7	M	17				Drowning
1500	Control		6.9	M	18				Motor vehicle accident
4898	Control		7.7	M	12		Hyperactive disorder	Concerta, Clonidone	Drowning
1708	Control		8.1	F	20				Motor vehicle accident
1706	Control		8.6	F	20		Congenital heart disease with heart transplant		Rejection of cardiac allograft transplantation
1407	Control		9.1	F	20		Asthma allergies	Albuterol, Zirtec, Alegra, Rodact, Flovent, Flonase	Asthma
4722	Control		14.5	M	16				Motor vehicle accident
1846	Control		20.6	F	9				Motor vehicle accident
4645	Control		39.2	M	12				Arteriosclerotic heart disease

ADI-R: Autism Diagnostic Interview Revised.
ADOS: Autism Diagnostic Observation Scale.
VABS: Vineland Adaptive Behavioral Scale.
BSID-II: Bayley Scales of Infant Development-Second Edition.
CARS: Childhood Autism Rating Scale.
WISC-R: Wechsler Intelligence Scale for Children-Revised.
According to the medical histories for UMB-4231 and UMB-5027, the donors had psychological evaluation, and met the criteria for a diagnosis of autism. Detailed information is not available.

Table 2. Autism Diagnostic Interview-Revised test scores in donors of brain tissue samples.

Autism Diagnostic Interview-Revised (ADI-R)[a]							
Diagnostic Algorithm	Cutoff score for autism	UMB 4671	UMB 4849	UMB 1174	UMB 797	UMB 4899	UMB 1638
Abnormalities in reciprocal social interaction (Scores:0-30)	10	26	22	22	24	22	21
Abnormalities in communication:							
Verbal (Scores:0-26)	8	-	18	-	20	-	-
Non-verbal (Scores: 0-14)	7	13	N/A	11	13	14	11
Restricted, repetitive and stereotyped patterns of behavior (Scores: 0-12)	3	3	8	6	6	8	7
Abnormalities of development evident at or before 36 months	1	5	3	5	-	4	5

a: Higher score represents greater impairment.
UMB 1182: ADI-R was conducted but the scores are not available. The donor met the criteria for a diagnosis of autism.

Table 3. Vineland Adaptive Behavioral Scales diagnostic test for autism in donors of brain tissue samples.

Vineland Adaptive Behavioral Scales (VABS)[a]							
	UMB 1349				UMB 4671		UMB 1174
	At age: 25 months		At age: 33 months		At age: 39 months		At age: 6.4 y
Domain (Scores:20-160)	Standard Score	Age equivalent performance	Standard Score	Age equivalent performance	Standard Score	Age equivalent performance	Standard score
Communication	57	9 months	69	18 months	52	10 months	41
Daily living skills	65	16 months	62	16 months	54	14 months	22
Socialization	60	9 months	71	17 months	51	4 months	52
Motor skills	-	-	-	-	65	24 months	-
Composite	-	-	-	-	51	13 months	35

a: Higher score represents better function.

Preparation of brain homogenates. The post-mortem brain tissue samples from regressive autism, non-regressive autism, and control subjects were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitor cocktail at 4°C. For extraction of protein kinases, the homogenates were mixed with an equal volume of extraction buffer containing 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM Na_3VO_4, 100 mM NaF, and 2 µg/ml leupeptin. The samples were allowed to stand on ice for 10 minutes, and then centrifuged at 135,000 g for 20 minutes at 4°C. The supernatants were collected, and stored at –70°C. The concentrations of total proteins in the supernatants were measured by the biocinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL).

Assay of PKC activity. The activity of PKC in the brain supernatants was measured by solid phase enzyme-linked immuno-absorbent assay (ELISA) kit from Enzo Life Sciences International, Inc. The assay is designed for the analysis of PKC activity in the solution phase. In this assay, microplates pre-coated with PKC substrate were used. The microplate wells were soaked with dilution buffer and were emptied after 10 minutes. An equal volume of the brain supernatants was added to the wells, followed by the addition of ATP to initiate the reaction. After incubation for 90 minutes at 30°C, the kinase reaction was terminated by emptying the contents of each well. The phosphopeptide substrate thus obtained was immunodetected by using phospho-substrate specific primary antibody and peroxidase-conjugated secondary antibody as per manufacturer's instructions. The mean absorbance ($x10^3$) of samples was divided by the quantity of total protein (µg) used per assay, and the data is represented as relative PKC activity.

Statistical analysis. Initially, autistic and control cases were collected as age-matched pairs. As data for both members of a pair were not available in all cases,

and data were approximately normally distributed, unpaired two-tailed t-tests were employed to make comparisons of PKC activity in various brain regions between autistic vs. control cases. The data was considered significant if 'p' was < 0.05. Comparisons among controls and autistic cases showing or not showing clinical signs of regression in function were made using one-way analysis of variance (ANOVA). To guard against Type I error, a Bonferroni adjustment for multiple comparisons was made to the t-tests of multiple brain regions, and for the pair wise post-hoc t- tests comparing each pair of the three groups that were compared in the overall ANOVA. Data is presented as Mean ± S.E.

Pearson's correlation coefficient (r) was used to evaluate if there was relationship between PKC activity in autism and behavioral abnormalities (ADI-R score).

RESULTS

PKC activity in the cerebellum and different cerebral regions of brain from subjects with regressive autism, non-regressed autism and their age-matched controls.

PKC activity was assayed in the frontal, temporal, occipital and parietal cortices (Fig. 1), and cerebellum (Fig. 2) from subjects with autism (regressive and non-regressive) and their age-matched control subjects. As shown in Fig. 1, PKC activity was significantly decreased by 65.8% (p = 0.0048) in the frontal cortex of subjects with regressive autism (Mean ± S.E.; 2.05 ± 0.41) as compared to non-regressive autistic subjects (Mean ± S.E.; 6.00 ± 0.97), and by 57.1% (p = 0.0085) as compared to age-matched control subjects (Mean ± S.E.; 4.78 ± 0.63). On the other hand, PKC activity was similar between non-regressive autism and age-matched control groups. We also analyzed the data with one-way ANOVA test using Bonferroni adjustment for multiple comparison, and observed that data was significant (p = 0.0057). Alteration of PKC activity in the frontal cortex of subjects with regressive autism was brain regions-specific. PKC activity was not affected in other brain regions i.e., cerebellum, and in the temporal, occipital and parietal cortices from autism subjects (regressive and non-regressive autism) as compared to age-matched controls (Figs. 1 and 2).

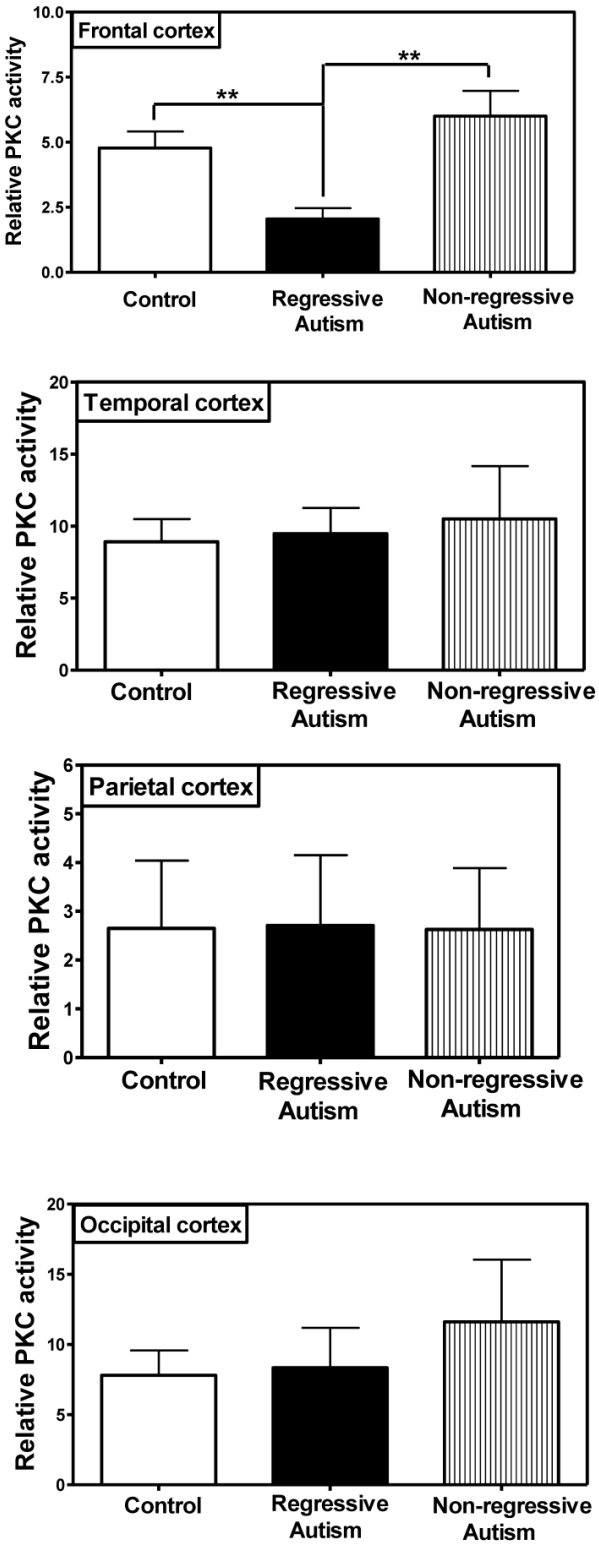

Fig. 1. Protein kinase C activity in different regions of cerebral cortex, i.e., frontal, temporal, occipital and parietal cortex from subjects with regressive autism, non-regressed autism and their age-matched controls. The mean absorbance ($x10^3$) of samples was divided by the quantity of total protein (μg) used per assay, and the data is represented as relative PKC activity. **p < 0.01 as compared to control and non-regressed autism groups.

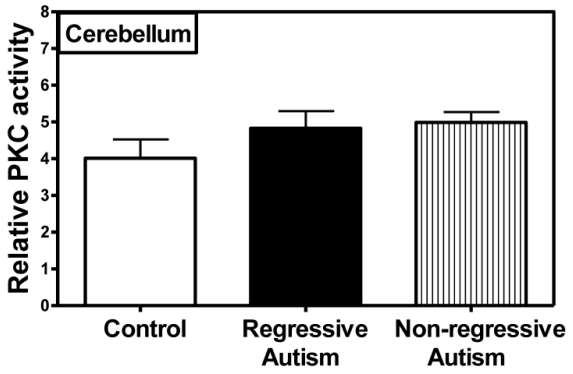

Fig. 2. Protein kinase C activity in the cerebellum from subjects with regressive autism, non-regressed autism and their age-matched control subjects. The mean absorbance (x10³) of samples was divided by the quantity of total protein (µg) used per assay, and the data is represented as relative PKC activity.

Postmortem interval (PMI) and age of the subjects. There was no significant difference in age (mean ± S.E.) of the subjects among the regressive autism (11.6 ± 2.7 years), non-regressive autism (13.7 ± 6.1 years) and control groups (12.4 ± 3.3 years). Our results also suggest that PMI was not a contributing factor to the observed alteration in PKC activity in the frontal cortex of individuals with regressive autism because PKC activity in the cerebellum and the temporal, parietal, and occipital cortices was not affected in subjects with regressive autism in comparison with control subjects, while it was affected only in the frontal cortex from these individuals with regressive autism.

Correlation of PKC activity with behavioral abnormalities in autism. In order to evaluate whether there is any correlation between reduced PKC activity and behavioral abnormalities in subjects with autism, we analyzed the data of PKC activities in the frontal cortex as a function of ADI-R scores for different behavioral parameters (Fig. 3). In this study, we had ADI-R scores of only six subjects with autism, which included three regressive autism subjects (UMB # 4849, 4899, 1638) and three non-regressive autism subjects (UMB # 4671, 1174, 797). Fig. 3a shows the correlation between PKC activity in the frontal cortex and ADI-R score for restrictive, repetitive and stereotyped behavior in regressive and non-regressive autistic subjects. It was observed that ADI-R test score for restrictive, repetitive and stereotyped pattern of behavior was higher in regressive autism as compared to non-regressive autistic subjects. Interestingly, linear regression analysis showed a negative significant correlation between PKC activity in the frontal cortex and restrictive, repetitive and stereotyped behavior (r = –0.84, p =0.0363). A comparison of PKC activity in combined regressive and non-regressive autism group with ADI-R scores for abnormalities of development before the age of 36 months did not show a correlation between these two parameters (data not shown). However, a negative correlation (r = –0.988) in subjects with regressive autism was observed between PKC activity and abnormalities of development before the age of 36 months, though it did not reach significance (p =0.09, n=3) (Fig. 3b). On the other hand, there was no correlation between reduced PKC activity and impairments in reciprocal social interaction in regressive or non-regressive autistic subjects (data not shown). Abnormalities in communication had two types of scores: verbal and non-verbal. Only two scores were available in verbal category, which were not sufficient for analysis. Therefore, we analyzed the PKC data in the frontal cortex with respect to non-verbal score, and did not find any significant correlation between these parameters in regressive or non-regressive autism (data not shown).

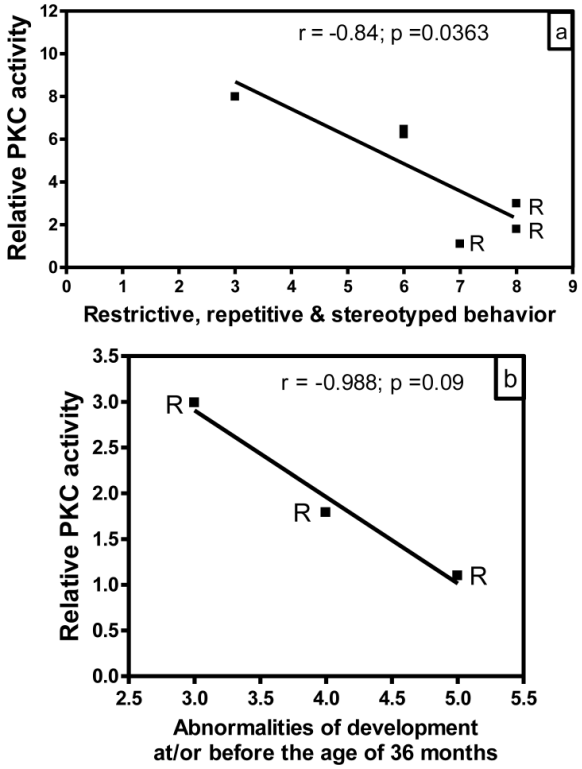

Fig. 3. Relationship between PKC activity of frontal cortex and Autism Diagnostic Interview Revised (ADI-R) test scores in subjects with autism. PKC activity was plotted against individual ADI-R scores for (a) restricted, repetitive and stereotyped patterns of behavior, and (b) abnormalities of development evident before the age of 36 months. R represents subjects with regressive autism.

DISCUSSION

Autism is a multifactorial disorder with variability in many domains. The variability of domains includes high or low functioning autism, regressive or non regressive autism, and comorbidities such as epilepsy. No single factor can explain variability observed with different domains of autism. Our results are suggestive of reduced PKC activity in the frontal cortex as one of the factors contributing to regression in autism. Recently, we reported that PKA is also affected in the frontal cortex of subjects with regressive autism [33]. Collectively, our present results and previous report suggest that regression in autism may be the result of alterations in protein kinases-mediated signal transduction. It is possible that both of these kinases (PKC and PKA) are affected by a common pathway because both protein kinases are activated by G-protein-coupled receptors. PKA gets activated by G-protein-coupled adenyl cyclase that converts ATP to c-AMP, an activator of PKA. On the other hand, activation of PKC is associated with G-protein-coupled phospholipase C-mediated cleavage of phosphoinositides into two intracellular messengers, i.e., diacylglycerol (DG) (activator of PKC) and inositol trisphosphate (IP_3) (a Ca^{2+} mobilizer).

In the brain, the signals that control cognition vary depending on type of G-protein-coupled signal input. Several receptors such as glutamatergic receptors [37], cholinergic receptors [38], serotonergic receptors [39] and dopaminergic receptors [40] regulate the functions of PKC. PKC is a key regulator of neuronal signal transduction pathways that are crucial to learning and memory consolidation [41-45]. Neuronal plasticity and synaptic connections are important for information processing in the brain. Activation of PKC facilitates synaptic plasticity that includes responses such as Ca^{2+} influx, neurotransmitters release, and a decrease in Ca^{2+}-activated K current in the brain, leading to the enhancement of neuronal excitability and potentiation of synaptic response [46; 47]. Li et al. [48] also reported effect of chronic treatment with staurosporine (PKC-inhibitor) on acquisition and expression of contextual fear conditioning in rats. Considering the importance of PKC in neuronal functions, decreased PKC activity in subjects with regressive autism may result in decreased neuronal plasticity, thus affecting neuronal excitability and synaptic response.

The formation of functional neuronal synapse requires various molecular players in presynaptic and postsynaptic growth. Dysfunction of proteins such as neuroligins, neurexin and SHANK that are required for synaptic development have been reported in ASDs [49; 50]. Neural disconnection leading to abnormalities in cortical networks has been suggested in autism. Different isozymes of PKC are known to have important roles at various stages of brain development. Purkayastha et al. [51] reported that serotonin 1A receptor-mediated signaling during neonatal hippocampal development initially requires PKCε to boost neuronal proliferation, and then uses PKCα to promote synaptogenesis.

Our results also suggest a relationship of reduced PKC activity in the frontal cortex with some behavioral abnormalities in autism. According to ADI-R diagnostic algorithm criteria, higher score reflects greater behavioral impairment. A negative significant relationship was observed between PKC activity in the frontal cortex and restrictive, repetitive and stereotyped behavioral score in autistic subjects ($r= -0.84$). A negative correlation was also observed between PKC activity in the frontal cortex and abnormalities of development before the age of 36 months in regressive autistic subjects ($r= -0.988$). However, later correlation did not reach significance, which may be due to small sample size in this study. The correlation between reduced PKC activity in the frontal cortex and behavioral abnormalities in autism needs further validation with larger sample size.

The prefrontal cortex has been implicated in autism to explain deficits in brain functions related to cognition, language, sociability and emotion [52]. Our findings of decreased activities of PKC and PKA in the frontal cortex of subjects with regressive autism suggest defective phosphorylation/ dephosphorylation of proteins. Both PKC and PKA are involved in neuronal signal transduction. Chronic treatment with carbamazepine (a mood-stabilizer drug) has been reported to increase phosphorylation of myristoylated alanine-rich C kinase substrate (MARKS) in the rat cerebral cortex, suggesting involvement of PKC –mediated phosphorylation of MARKS in behavioral changes [53]. A recent study showed that PKA inhibitor could induce behavioral and neurological antidepressant-like effects in rats [54]. Since both PKC and PKA are activated by G-proteins-coupled receptors and are extensively involved in brain functions, we suggest that inhibition of these kinases in the cerebral cortex may have significant role in regressive autism.

Autism belongs to a group of neuropsychiatric disorders. The roles of PKC and PKA have also been suggested in other neuropsychiatric disorders. Decreased protein expression of PKCβ1, PKCξ and PKA regulatory Iα subunit and PKA catalytic subunits (α and β) has been reported in the postmortem brain samples from major depressive subjects as compared

to controls [55]. Alterations in PKC activity were reported in manic depression, and antimanic agents (lithium carbonate and sodium valproate) inhibited PKC-associated signaling in brain tissue [56]. In other studies, prenatal and postnatal exposures to valproic acid (antiepileptic drug) have been used for animal model of autism to induce behavioral and neuropathological abnormalities similar to those observed in individuals with autism [57-61]. In pediatric bipolar disorder, decreased expression of specific PKC isozymes and decreased PKC activity in the platelets were reported [62]. PKC has also been suggested as a molecular target in pathogenic and therapeutic mechanisms of mood disorders in which electroconvulsive seizure (ECS) is effective [63]. This group reported phosphorylation of PKC substrates, including GAP-43, myristoylated alanine-rich C-kinase substrate, and neurogranin in the brain of rats after ECS. Another study showed significant decrease in the activities of phospholipase C and PKC in the membrane and cytosolic fractions of platelets from patients with bipolar disorder, suggesting that PKC may be involved in the pathophysiology of bipolar disorder [64].

The involvement of PKC has also been reported in other conditions such as inflammation [65], immune disorders [66], and oxidative stress [67]. These studies have suggested inhibitors of PKC theta as anti-inflammatory therapeutic agents [65], and PKC isozymes as potential therapeutic targets in immune disorders [66]. Abnormalities in inflammation, immune system and oxidative stress have been observed in autism [7]. Several lines of evidence from our and other groups have shown increased oxidative stress damage coupled with reduced antioxidant defense in blood [3; 6; 11; 14], brains [8-10; 13] and urine [12] of subjects with autism. We and others have also reported increased inflammatory markers in autism [17-21]. Therefore, PKC may also have a role in inflammation, immune defects and oxidative stress observed in autistic individuals.

Our results suggest that PMI and age cannot account for the observed alteration in PKC activity in subjects with regressive autism. Other factors, such as comorbidity with seizure disorder, reported for three of 10 autism cases (of which two had regressive autism, and one had non-regressive autism), and medications, reported for two regressive autism cases, four non-regressive autism cases, and two control cases, do not seem to be contributing factors to the altered activity of PKC in regressive autism. Furthermore, PKC activity was affected only in the frontal cortex but not in other brain regions of subjects with regressive autism. However, further studies with a larger autistic group should be done to explore this issue.

Considering the central role played by PKC in cellular signaling, the present findings on reduced PKC activity in subjects with regressive autism may result in disruption of neuronal signal transduction pathways in the frontal cortex, which may, in part, be responsible for regression in autism. It will be interesting to conduct a detailed study on the relationship between PKC activity and behavioral abnormalities with larger number of samples from subjects with autism. In conclusion, our study suggests that brain region-specific reduced PKC activity in the frontal cortex of individuals may be associated with regressive autism.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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