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Increased activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase in the frontal cortex and cerebellum of autistic individuals

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Abstract

Aims—Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase are enzymes known to maintain intracellular gradients of ions that are essential for signal transduction. The aim of this study was to compare the activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase in post-mortem brain samples from the cerebellum and frontal, temporal, parietal, and occipital cortices from autistic and age-matched control subjects.

Main methods—The frozen postmortem tissues from different brain regions of autistic and control subjects were homogenized. The activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase were assessed in the brain homogenates by measuring inorganic phosphorus released by the action of Na⁺/K⁺- and Ca²⁺/Mg²⁺- dependent hydrolysis of ATP.

Key findings—In the cerebellum, the activities of both Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase were significantly increased in the autistic samples compared with their age-matched controls. The activity of Na⁺/K⁺-ATPase but not Ca²⁺/Mg²⁺-ATPase was also significantly increased in the frontal cortex of the autistic samples as compared to the age-matched controls. 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.

Significance—The results of this study suggest brain-region specific increases in the activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase in autism. Increased activity of these enzymes in the frontal cortex and cerebellum may be due to compensatory responses to increased intracellular calcium concentration in autism. We suggest that altered activities of these enzymes may contribute to abnormal neuronal circuit functioning in autism.

INTRODUCTION

Autism is a severe neurological disorder that causes impairment in language, cognition and socialization (Lord et al. 2000). It is a heterogeneous disorder, both etiologically and phenotypically. Autism belongs to a group of neurodevelopmental disorders known as autism spectrum disorders (ASD) that includes Pervasive Developmental Disorder- Not Otherwise Specified (PDD-NOS), Asperger disorder, Childhood Disintegrative Disorder (CDD) and Rett syndrome. According to the Centers for Disease Control (CDC), 1 in 150 children is diagnosed with ASD.

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Genetic, neurochemical, neuroimaging and behavioral studies suggest that neural properties may be perturbed in autism, giving rise to abnormalities in processing of neuronal information leading to complex behavioral abnormalities (Belmonte et al., 2004). Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase are known to play important roles in neuronal transmission. A gradient of high K^+ and low Na^+ intracellular concentration is needed for the optimum neuronal functions (McCormick and Huguenard, 1994, Palladina et al., 2005). Na^+/K^+ -ATPase is a membrane-bound enzyme involved in maintaining the Na^+ and K^+ gradient across the cell membrane. It is ubiquitously expressed in neurons (Pietrini et al., 1992), and helps maintain normal neuronal function. The Na^+/K^+ -ATPase extrudes three Na^+ ions and imports two K^+ ions. This activity is important in the regulation of membrane potential. The Na^+/K^+ -ATPase activity contributes to the resting membrane potential in the cell, and returns Na^+ and K^+ concentrations to their resting transmembrane levels after bursts of stimulatory activity (Blaustein, 1993). Na^+/K^+ -ATPase abnormality has been reported to be involved in several neurological diseases such as seizures (Brines et al., 1995; Fernandes et al., 1996b), bipolar disorder (Amiet et al., 2008; Christo and el Mallakh, 1993), spongiform encephalopathy (Renkawek et al., 1992), and Alzheimer's disease (Rose and Valdes, 1994). Na^+/K^+ -ATPase may also have implications in behavioral defects. Lingrel et al. (2007) reported that haploinsufficiency of Na^+/K^+ -ATPase $\alpha 2$ and $\alpha 3$ isoforms results in behavioral defects.

Calcium is an important signaling molecule in cells (Berridge, 1992). Many cellular functions are regulated by intracellular free calcium concentrations. In resting cells, a sub-optimum concentration of intracellular calcium is maintained either by storing calcium in intracellular reserves by the action of ATPase (Nori et al., 1996), by extrusion of calcium by plasma membrane-bound calcium ATPase (Carafoli et al., 1996) or by $\text{Na}^+/\text{Ca}^{2+}$ exchange (Blaustein and Lederer, 1999). In stimulated cells, a sudden influx of calcium occurs in a receptor-coupled manner where calcium participates in activating several proteins, which perform specific functions. Neurons use intracellular Ca^{2+} to control various functions. Disturbances in Ca^{2+} homeostasis can lead to neuronal dysfunction and eventual neuronal death. Several neurological diseases are caused primarily by malfunctioning of Ca^{2+} channels or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase (Cooper and Jan, 1999; Jacobsen et al., 1999). Recently, Gargus reported genetic calcium signaling abnormalities in several neurological conditions, including seizures, migraines and autism (Gargus, 2009). However, it is not known whether Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase abnormalities are involved in autism. The present study was undertaken to determine whether the activities of Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase are affected in autism. We found that activities of Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase are specifically increased in the frontal cortex and cerebellum of brains from autistic subjects, while they were unchanged in the parietal, occipital and temporal cortices.

MATERIALS AND METHODS

Materials

Samples of postmortem frozen brain regions, i.e., the cerebellum, and cortices from the frontal, temporal, parietal and occipital lobes ($N = 6-10$ for different brain regions) from autistic and age-matched control subjects ($N = 8-10$) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders. The age (Mean $\pm$ S.E.) for autistic subjects was 13 ± 3.7 y and for control subjects, 12.5 ± 3.5 y. The mean postmortem interval (PMI) for the autistic samples was 22 ± 4.5 h, and for control samples, 17 ± 1.3 h. Donors with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV, as confirmed by the Autism Diagnostic Interview-Revised (ADI-R). 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.

Preparation of homogenates

The tissues 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) in a Downs homogenizer with 5 strokes at 4 °C. The protein concentration was assayed by the BioRad protein assay kit.

Measurement of Na⁺/K⁺-ATPase activity

The reaction mixture containing 25 μ l of 2 M NaCl, 25 μ l of 25 mM KCl, 25 μ l of 60 mM MgCl₂, 5 μ l of 10 mM EGTA and brain homogenate (0.5 mg) was adjusted to a total volume of 490 μ l with 50 mM Tris-HCl, pH 7.5, and incubated at 37 °C for 10 min. The reaction was started by adding 10 μ l of 150 mM ATP. After 1 h, the reaction was stopped by adding 1 ml of cold 15% TCA. The samples were kept on ice for 1 h, followed by centrifugation at 1,000 g for 15 min. Inorganic phosphorus in the 500 μ l supernatant was measured by the method of Fiske and Subbaraw (1953).

Measurement of Ca²⁺/Mg²⁺-ATPase activity

The reaction mixture containing 25 μ l of 2 M NaCl, 25 μ l of 25 mM KCl, 25 μ l of 60 mM MgCl₂, 5 μ l of 10 mM Quabain, 10 μ l of 10 mM CaCl₂, and brain homogenate (0.5 mg) was adjusted to a total volume of 490 μ l with 50 mM Tris-HCl, pH 7.5, and incubated at 37 °C for 10 min. The reaction was initiated by adding 10 μ l of 150 mM ATP. After 1 h, the reaction was stopped by adding 1 ml of cold 15% TCA. The samples were kept on ice for 1 h, followed by centrifugation at 1,000 g. Inorganic phosphorus in the 500 μ l supernatant was then measured.

Statistical analysis

The enzyme activities in autism and control groups were analyzed by unpaired student's t-test.

RESULTS

Na⁺/K⁺-ATPase activity in different brain regions from autistic and control subjects

Fig. 1 shows the Na⁺/K⁺-ATPase activity in the cerebellum of autistic and age-matched control subjects. The Na⁺/K⁺-ATPase activity (μ g phosphorus released/mg protein/hr) in the cerebellum of the autistic samples (Mean $\pm$ S.E.: 188 ± 13) was significantly higher ($p < 0.03$) as compared to control samples (156 ± 8). In Fig. 2, the Na⁺/K⁺-ATPase activity in the cortices from frontal, temporal, parietal and occipital regions is shown. The activity of Na⁺/K⁺-ATPase in the frontal cortex of the autistic subjects (169 ± 5) was significantly higher ($p < 0.03$) as compared to the control subjects (145 ± 8.7). However, the activity of the Na⁺/K⁺-ATPase was similar between the autistic and control groups in other brain regions, i.e., temporal cortex (autism, 142 ± 13 ; and controls, 128 ± 7.5); parietal cortex (autism, 131 ± 4 ; and controls, 130 ± 6); and occipital cortex (autism, 294 ± 22 , and controls, 309 ± 15). No relation was observed between PMI and the activity of this enzyme in the brain.

Ca²⁺/Mg²⁺-ATPase activity in different brain regions from autistic and control subjects

Ca²⁺/Mg²⁺-ATPase activity in the cerebellum of autistic and control subjects is shown in Fig. 3. The Ca²⁺/Mg²⁺-ATPase activity (μ g phosphorus released/mg protein/hr) in the cerebellum of the autistic samples (Mean $\pm$ S.E.: 190 ± 13) was significantly higher ($p < 0.0005$) than in age-matched controls (130 ± 4.5). There was no overlap in the Ca²⁺/Mg²⁺-ATPase activity between autistic and control samples. Fig. 4 shows the Ca²⁺/Mg²⁺-ATPase activity in the cortices from frontal, temporal, parietal and occipital regions. No significant differences in the activity of Ca²⁺/Mg²⁺-ATPase was observed in frontal cortex (autism, 105 ± 6.7 ; and controls, 96.7 ± 5.6); temporal cortex (autism, 110 ± 12 ; and controls, 98 ± 6); parietal cortex (autism,

111 ± 6, and control, 115 ± 10); and occipital cortex (autism, 211 ± 22; and controls, 200 ± 8). No relation was observed between PMI and this activity of the enzyme in the brain.

DISCUSSION

K⁺, Na⁺ and Ca²⁺ play important roles in neuronal signaling due to conduction of electrical activity of neurons (McCormick and Huguenard, 1994). Therefore, control of excitability of neurons is maintained by the ionic environment. Intracellular concentrations of Na⁺ and Ca²⁺ are several folds lower and that of potassium are higher as compared to their extracellular concentrations. The net transmembrane potential across the membrane is maintained at −60 mV. If the ionic concentration is perturbed (e.g., levels of intracellular Ca²⁺ and Na⁺ or extracellular K⁺ are altered), this can lead to depolarization and abnormal neuronal activity due to depolarization of neuronal-terminals, neurotransmitters release, depolarization of neurons and discharge of action potential (Somojen, 2002).

Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase are two ATP-hydrolyzing enzymes which maintain the electrochemical gradient in the cells in an energy-dependent manner. Na⁺/K⁺-ATPase extrudes three Na⁺ molecules in exchange for internalization of two K⁺ molecules. The Na⁺/K⁺-ATPase is composed of multiple isoforms (α1, α2 and α3), and these isoforms differ in their distribution in tissues and during development. Lingrel et al. (2007) reported that haploinsufficiency of its α2 and α3 isoforms results in behavioral defects. In another study, mutations in a C-terminal region of other voltage-gated Na⁺ channels have been reported to reduce the amount of channel inactivation (Glaaser et al., 2006; Kim et al., 2004). Another report suggests functional deficit of Ca²⁺-activated K⁺ channel (BKCa), a synaptic regulator of neuronal excitability with autism (Laumonnier et al., 2006). Disruption of the BKCa gene (KCNMA1) led to haploinsufficiency and reduced BKCa activity in autism. These reports on decrease in BKCa channel activity, and reduced inactivation of voltage-gated Ca²⁺ channels in individuals with autism, raise the possibility that excessive ion channel activity may lead to ASD.

Since K⁺, Na⁺ and Ca²⁺ play important roles in developing electrochemical gradients and in neuronal signaling, the altered activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase may have a significant impact on brain function in autistic subjects. Our results show that the activities of both Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase were significantly increased in the cerebellum in autism as compared with age-matched controls, while the activity of Na⁺/K⁺-ATPase was also significantly increased in the frontal cortex in autism. In other regions of cerebrum i.e., occipital, parietal and temporal cortex, the activities of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase were similar between autistic and control subjects. Increased activity of Na⁺/K⁺-ATPase has been reported in several other pathological conditions such as in experimentally induced epilepsy (Fernandes et al., 1996; Reime et al., 2007), and in Crush syndrome (Desai and Desai, 2007). In chronic fatigue syndrome, the activities of both Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase are increased in sarcoplasmic reticulum membranes (Fulle et al., 2003). In addition, Takser et al. (2003) reported a correlation of ATPase activities with early psychomotor development in humans. Rapid eye movement sleep deprivation has also been reported to increase Na⁺/K⁺-ATPase activity (Mallick et al., 2000). In addition, certain environmental factors such as lead have been reported to increase the activity of Na⁺/K⁺-ATPase (Regunathan and Sundaresan, 1985).

After a stimulus, calcium flows rapidly into neurons through various types of membrane channels including voltage-dependent and receptor-coupled channels. Intracellular Ca²⁺ concentrations are quickly restored to resting levels primarily through Ca²⁺/Mg²⁺-ATPase, Na⁺/Ca⁺ exchange, and endoplasmic sequestration. Calcium is essential for neurotransmitter release, and Ca²⁺ influx is essential for neuronal excitability. Improper intracellular regulation

of calcium has been linked with several neurological disorders. The receptor-coupled increase in intracellular levels of calcium is important for neuronal survival, differentiation, migration, and synaptogenesis (Aamodt and Constantine-Paton, 1999; Cline, 2001; Komuro and Rakic, 1998; Moody and Bosma, 2005; Represa and Ben Ari, 2005; Spitzer et al., 2004). Defects in these developmental processes can lead to neuroanatomical abnormalities, such as increased cell-packing density, decreased neuron size and arborizations, and alterations in connectivity. Such abnormalities have been associated with ASD patients (Courchesne et al., 2005; DiCicco-Bloom et al., 2006). Plasma membrane calcium-ATPase plays an important role in the translocation of calcium from the cytosol to the extracellular milieu. Our results suggest that $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity is significantly increased in the cerebellum of autistic subjects, but not in other regions of the brain. Although $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity in the frontal cortex of autism subjects was not significantly changed but a trend towards increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity was observed as compared to controls. The median $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity in the frontal cortex for autism was 113 μg phosphorus released/mg protein/hr while for control group, it was 99 μg phosphorus released/mg protein/hr. A differential effect of ATPase activity in different regions of brain is not unique. In epilepsy, the intrasynaptosomal $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity was reported to be decreased in the hippocampus, but not in the temporal cortex (Nagy et al., 1990).

Voltage-gated calcium channels mediate calcium influx in response to membrane depolarization and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression. Their activity is essential for coupling electrical signals on the cell surface to physiological events in cells. Functional mutations in genes encoding voltage-gated Ca^{2+} channels have been suggested as a possible cause of ASD (Hemara-Wahanui et al., 2005b; Splawski et al., 2006; Splawski et al., 2004). Point mutations in the gene encoding the L-type voltage-gated Ca^{2+} channel $\text{CaV}1.2$ (*CACNA1C*) cause Timothy syndrome, a multisystem disorder that includes cardiac abnormalities and autism (Splawski et al., 2005; Splawski et al., 2004). $\text{CaV}1.2$ plays an important role in the activation of transcription factors, such as cAMP response-element-binding protein (CREB) and myocyte enhancer factor 2 (MEF2), involving neuronal survival and dendritic arborization (West et al., 2001). The mutations associated with Timothy syndrome prevent voltage-dependent inactivation of $\text{CaV}1.2$, which causes the channels to remain open longer and allow the influx of more Ca^{2+} than wild-type channels (Splawski et al., 2005; Splawski et al., 2004) leading to increased intracellular Ca^{2+} . Additional evidence of calcium's involvement in autism comes from a mutation identified in the *CACNA1F* gene, which encodes the L-type voltage-gated Ca^{2+} channel, $\text{CaV}1.4$. This mutation was reported to cause autistic symptoms in a New Zealand family where the affected subjects have stationary night blindness (Hemara-Wahanui et al., 2005a; Hope et al., 2005). ASD-associated mutations have been identified not only in genes encoding Ca^{2+} channels themselves but also in genes encoding ion channels whose activity is directly modulated by Ca^{2+} such as Ca^{2+} -dependent Na^{+} channels. Several point mutations in *SCN1A* and *SCN2A* genes, which encode the voltage-activated Na^{+} channels $\text{NaV}1.1$ and $\text{NaV}1.2$ respectively has been reported (Kamiya et al., 2004; Weiss et al., 2003).

Wingless-type mouse mammary tumor virus (MMTV) integration site member (Wnt) proteins are known to form a family of highly conserved and secreted signaling molecules, which regulate cell-to-cell interactions during embryogenesis. The role of WNT2 has been implicated in ASD. Two families with mutations in WNT2 have been identified, and a polymorphism in an upstream region of WNT2 has been associated with families characterized with severe language abnormalities (Wassink et al., 2001). Increase in Ca^{2+} concentration has been reported to enhance the synthesis and release of Wnt through the activity of the Ca^{2+} -regulated transcription factor CREB (Wayman et al., 2006). Because of the pivotal role of calcium in cellular signaling, calcium may play an important role in the etiology of ASD.

The increased activity of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase in the cerebellum of autistic subjects may be attributable to several factors. $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity may increase due to compensatory mechanisms in response to increased intracellular calcium levels in autism. Heguilen et al. (2009) reported increases in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity in patients with hypercalciuric nephrolithiasis. In addition, $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity can also be activated by lysophosphatidylcholine, a phospholipase A₂ (PLA₂)–mediated lipolytic product in the membrane. It has also been reported that the levels of polyunsaturated fatty acids, another lipolytic product of PLA₂, are decreased in the erythrocyte membranes of autistic subjects as compared with normal control subjects (Bell et al., 2000). Increased activity of PLA₂, an enzyme that removes unsaturated fatty acids from phospholipids, has also been reported in erythrocytes from autistic subjects (Bell et al., 2004). Additionally, increased levels of phospholipase A₂ have been observed in the erythrocytes of patients with schizophrenia (Ward, 2000) and dyslexia (MacDonell et al., 2000). Since chromosomal linkage studies in autism point to a locus which includes the PLA₂ gene (Lamb et al., 2000), this enzyme may also have an important role in the etiology of autism. In conclusion, $\text{Na}^{+}/\text{K}^{+}$ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activities in autism may be increased in response to increased intracellular calcium concentration, and may contribute to altered neocortical circuitry in the cerebellum and frontal cortex of individual with autism.

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Fig. 1. Na⁺/K⁺-ATPase activity in the cerebellum of autistic and control subjects

The enzyme activity was measured in the cerebellum of autistic and control subjects as described in 'Materials and Methods'. The horizontal line represents average Na⁺/K⁺-ATPase activity in each group. * denotes $p < 0.05$, autism vs. control group.

Fig. 2. Na⁺/K⁺-ATPase activity in the frontal, occipital, parietal and temporal cortices from autistic and control subjects

The activity of Na⁺/K⁺-ATPase was measured in different brain regions from autistic and control subject as described in 'Materials and Methods'. * denotes $p < 0.03$, autism vs. control group.

Fig. 3. Ca²⁺/Mg²⁺-ATPase activity in the cerebellum of autistic and control subjects
The enzyme activity was measured in the cerebellum of autistic and control subjects as described in 'Materials and Methods'. The horizontal line represents average Ca²⁺/Mg²⁺-ATPase activity in each group. * denotes $p < 0.0005$, autism vs. control group.

Fig. 4. $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity in the frontal, occipital, parietal and temporal cortices of the brain regions from autistic and control subjects
The activity of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase was measured in different brain regions from autistic and control subjects as described in 'Materials and Methods'.