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Abnormal partitioning of hexokinase 1 suggests disruption of a glutamate transport protein complex in schizophrenia

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Abstract

Excitatory amino acid transporter 2 (EAAT2) belongs to a family of Na⁺ dependent glutamate transporters that maintain a low synaptic concentration of glutamate by removing glutamate from the synaptic cleft into astroglia and neurons. EAAT2 activity depends on Na⁺ and K⁺ gradients generated by Na⁺/K⁺ ATPase and ATP. Hexokinase 1 (HK1), an initial enzyme of glycolysis, binds to mitochondrial outer membrane where it couples cytosolic glycolysis to mitochondrial oxidative phosphorylation, producing ATP utilized by the EAAT2/Na⁺/K⁺ ATPase protein complex to facilitate glutamate reuptake. In this study, we hypothesized that the protein complex formed by EAAT2, Na⁺/K⁺ ATPase and mitochondrial proteins in human postmortem prefrontal cortex may be disrupted, leading to abnormal glutamate transmission in schizophrenia. We first determined that EAAT2, Na⁺/K⁺ ATPase, HK1 and aconitase were found in both EAAT2 and Na⁺/K⁺ ATPase interactomes by immunoprecipitation and mass spectrometry in human postmortem prefrontal cortex. Next, we measured levels of glutamate transport complex proteins in subcellular fractions in the dorsolateral prefrontal cortex and found increases in the EAAT2B isoform of EAAT2 in a fraction containing extrasynaptic membranes and increased aconitase 1 in a mitochondrial fraction. Finally, an increased ratio of HK1 protein in the extrasynaptic membrane/mitochondrial fraction was found in subjects with schizophrenia, suggesting that HK1 protein is abnormally partitioned in this illness. Our findings indicate that the integrity of the glutamate

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transport protein complex may be disrupted, leading to decreased perisynaptic buffering and reuptake of glutamate, as well as impaired energy metabolism in schizophrenia.

Keywords

Excitatory amino acid transporter (EAAT); Dorsolateral prefrontal cortex (DLPFC); Reuptake; Postmortem; Immunoisolation; Mass spectrometry

1. Introduction

Schizophrenia is a serious psychiatric disease that afflicts over 2.4 million people in the USA and over 50 million people worldwide (Bhugra, 2005). The pathophysiology of schizophrenia likely includes abnormalities of glutamate transmission, since NMDA glutamate receptor antagonists such as phencyclidine (PCP) can induce the positive, negative and cognitive symptoms of this illness (Meador-Woodruff and Healy, 2000; Kristiansen et al., 2007; Bergeron and Coyle, 2012; Kantrowitz and Javitt, 2012). The effects of PCP might be due to increased release of glutamate at synapses secondary to the blockade of NMDA receptors, leading to abnormal modulation of non-NMDA subtype glutamate receptors (Marsman et al., 2013). The excitatory amino acid transporters (EAATs) play a critical role by limiting glutamate spillover from the synaptic cleft through the binding and transport of glutamate across the plasma membrane (Danbolt, 2001; Shigeri et al., 2004; Sheldon and Robinson, 2007; Tzingounis and Wadiche, 2007). Not surprisingly, recent work from animal models and postmortem studies has found changes in EAATs in schizophrenia (Ohnuma et al., 1998; Harrison, 1999; Ohnuma et al., 2000; Smith et al., 2001; McCullumsmith and Meador-Woodruff, 2002; Matute et al., 2005; Bauer et al., 2008; Walsh et al., 2008; Karlsson et al., 2009; Bauer et al., 2010; Rao et al., 2012; Shan et al., 2013).

EAATs are expressed on postsynaptic neurons and astrocytes throughout the brain, maintaining low glutamate levels at synapses (Chaudhry et al., 1995; Lehre et al., 1995; Furuta et al., 1997; Kugler and Schmitt, 1999; Shigeri et al., 2004; Sheldon and Robinson, 2007; Shan et al., 2012). EAAT1-2 are primarily localized to astroglia, and account for more than 90% of Na^+ dependent glutamate reuptake in the forebrain (Rothstein et al., 1994; Furuta et al., 1997; Shigeri et al., 2004; Sheldon and Robinson, 2007). With the process of glutamate reuptake, one glutamate molecule is transported into the cell with three Na^+ ions and one H^+ in exchange for one K^+ ion (Danbolt, 2001; Shigeri et al., 2004; Sheldon and Robinson, 2007). EAAT activity depends on an electrochemical gradient of high internal K^+ and low internal Na^+ generated by Na^+/K^+ ATPase and ATP (Blanco and Mercer, 1998; Rose et al., 2009; Larsson et al., 2010). The Na^+/K^+ ATPase is located on the plasma membrane and consists of a heterodimer of α and β subunits. The α subunit has four isoforms ($\alpha 1-4$), while the β subunit has three isoforms ($\beta 1-3$). The α subunit contains the binding sites for the cations, ATP, and the inhibitor ouabain. The β subunit is a glycosylated polypeptide that is essential for the activity of the enzyme (McDonough et al., 1990; Blanco and Mercer, 1998; Kaplan, 2002).

A number of animal studies have demonstrated that GLT-1 (called EAAT2 in human) interacts with Na^+/K^+ ATPase, regulating reuptake of glutamate in brain (Rose et al., 2009; Genda et al., 2011). Colocalization of Na^+/K^+ ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ with GLT-1 was observed in the CA1 region of the rat hippocampus and rat cortex by immunocytochemical analyses, coimmunoprecipitation and mass spectrometry (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011). It has been estimated that Na^+/K^+ ATPase consumes nearly half of the energy in the brain to maintain the electrochemical gradient of Na^+ and K^+ across cell membranes (Lipton and Robacker, 1983). The main energy source for the active complex of Na^+/K^+ ATPase and EAAT2 is provided by glycolysis (Parker and Hoffman, 1967; Lipton and Robacker, 1983; Balaban and Bader, 1984; Lynch and Balaban, 1987). Expression of HK1 overlaps with mitochondria and GLT-1 suggesting that GLT-1, mitochondria, and the first step in glycolysis colocalize in a protein complex (Genda et al., 2011). Taken together, these findings suggest that functional and efficient reuptake of glutamate from synapses relies on a transporter coupled protein complex; alterations in this complex may lead to abnormal glutamate transmission.

In this study, we first identified glutamate transport protein complex proteins in human postmortem prefrontal cortex by immunoisolation and mass spectrometry; we also measured levels of complex proteins in total homogenate and subcellular fractions in DLPFC from subjects with schizophrenia and a comparison group. Our studies test the hypothesis that the integrity of glutamate transporter protein complexes is compromised in schizophrenia.

2. Materials and methods

2.1. Subjects and tissue preparation

Human postmortem prefrontal cortex tissue used for immunoisolation, mass spectrometry, electron microscopy and blocking peptide experiments was provided by the Alabama Brain Collection. Brain tissues for our schizophrenia (fractionation and Western blot) studies were obtained from the Mount Sinai Medical Center brain collection. Subjects were diagnosed with schizophrenia based on DSM-III-R criteria. Subjects were excluded for a history of alcoholism, death by suicide, or coma for more than 6 h before death. Next of kin consent was obtained for each subject. Schizophrenia and comparison groups were matched for sex, age, pH, and PMI (Hammond et al., 2012). Comparison subjects were selected using a formal blinded medical chart review instrument with no history of psychiatric or neurological disease. Brain samples were obtained at autopsy from the left hemisphere. The DLPFC was dissected from Brodmann area 46. Samples were sectioned into 1 cm^3 pieces of tissue and stored at -80°C until further processing. Tissue was pulverized into a powder and stored at -80°C . 65 mg of pulverized sample from 16 pairs of schizophrenia and comparison subjects was suspended in 1.2 ml of isolation buffer containing 10 mM Hepes pH 7.8, 250 mM sucrose, 25 mM potassium chloride and 1 mM EGTA. The suspension was then transferred to a nitrogen cavitation chamber (Parr Instrument Company, Moline, IL, USA) and pressurized at 450 psi for 8 min for further disruption of cells. The homogenates were collected through the outlet port of the chamber by nitrogen decompression. A 60 μl aliquot was saved as total homogenate. The remainder was used for subcellular fractionation. All subjects were analyzed individually (they were not pooled).

For immunoisolation and mass spectrometry, 300 mg of postmortem prefrontal cortex tissue was homogenized by a Teflon-glass homogenizer in the cold homogenization buffer consisting of 5 mM Tris-HCl pH 7.4, 320 mM sucrose plus a protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany). After sonication using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) on ice at setting 3 for 5 s, the homogenates were then rotated on a shaker overnight at 4 °C with or without 1% SDS. The lysates were collected by centrifugation at 2300 g for 10 min. The protein concentration was then determined by a BCA protein assay kit.

2.2. Antibodies

Commercial primary antibodies were used as described below: Na⁺/K⁺ ATPase α 1 and pan Na⁺/K⁺ ATPase (1:500, Novus Biological, Littleton, Colorado, USA), EAAT2 (Western blot; 1:500, Millipore, Billerica, Massachusetts, USA), EAAT2 (Immunoisolation; 1:500, Santa Cruz Biotechnology, Santa Cruz, California, USA), hexokinase 1, and aconitase 1 and 2 (1:500, Abcam, Cambridge, Massachusetts, USA).

EAAT2B and exon 9 skipping form of EAAT2 (EAAT2 exon 9 skipping) antibodies were generated by GenScript (1:500, Piscataway, New Jersey, USA) by immunizing rabbit or goat, respectively. A unique 11 amino acid peptide-KHFPFMDIETCI at the C-terminal domain of human EAAT2B was used for generating EAAT2B antibody. The peptide of QIVTVRDRMRT which spans the splice region between exons 8 and 10 of EAAT2 was used for EAAT2 exon 9 skipping antibody.

2.3. Immunoisolation

Immunoisolation was performed using the pierce Crosslink Kit (Thermo Fisher Scientific, Rockford, Illinois, USA). 100 μ l of Protein A/G Plus Agarose beads was blocked with 1% BSA in 1 $\times$ PBS for 30 min at room temperature to reduce non-specific binding. After the beads were washed two times with 1 $\times$ coupling buffer, the washed beads were gently mixed with 10 μ g of anti EAAT2 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) or rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) and rotated at room temperature for 1 h. The antibody bound beads were washed 3 times with 1 $\times$ coupling buffer followed by one hour incubation with a DSS crosslinking solution at room temperature. The beads were then washed twice in elution buffer and twice in cold lysis/wash buffer. 1.5 ml of lysate was precleared by shaking with 200 μ l Pierce Control Agarose Resin at 4 °C for 1 h. 600 μ g of precleared lysate was then added to the beads bound with anti EAAT2 antibody or rabbit IgG control and gently mixed overnight at 4 °C. The complex of lysates, antibody or IgG control and beads was washed twice in lysis/wash buffer and once in 1 $\times$ conditioning buffer. The bound proteins were then eluted from the beads by incubating with 40 μ l of 2 $\times$ sample buffer at 70 °C for 10 min.

2.4. Sample preparation for mass spectrometry

Immunoisolation samples were loaded on 4–12% Bis-Tris gradient gels and stained with Simply Blue Coomassie (Invitrogen, Carlsbad, California, USA). The gel bands from the IgG or EAAT2 immunoisolation were excised above 50 kD and excess stain was removed by an overnight wash of 50% 100 mM ammonium bicarbonate/50% acetonitrile. After

destaining, disulfide bonds were reduced by 25 mM dithiothreitol at 50 °C for 30 min. Alkylation of the free thiol groups was carried out with 55 mM iodoacetamide for 30 min in the dark. The excess alkylating agent was removed and the gel pieces were washed twice with a 100 mM ammonium bicarbonate for 30 min. The gel pieces were evaporated to dryness in a SpeedVac (Savant) before the addition of the enzyme. 12.5 ng/μl of trypsin (Promega Gold Mass Spectrometry Grade) was added to each gel sample and incubated overnight at 37 °C. Peptides were extracted from the gel pieces using a 1:1 mixture of 5% formic acid and 50% aqueous acetonitrile twice for 15 min. Extracts were pooled and evaporated to dryness. The samples were then resuspended in 20 μl of a 0.1% formic acid prior to mass spectrometry analysis.

2.5. Nano liquid chromatography–tandem mass spectrometry

An aliquot (4 μl) of each digest was loaded onto a Nano cHiPLC 200 μm × 0.5 mm ChromXP C18-CL 3 μm 120 Å reverse-phase trap cartridge (Eksigent, Dublin, CA, USA) at 2 μl/min using an Eksigent autosampler. After washing the cartridge for 4 min with 0.1% formic acid in ddH₂O, the bound peptides were flushed onto a Nano cHiPLC column 75 μm × 15 cm ChromXP C18-CL 3 μm 120 Å (Eksigent, Dublin, CA, USA) with a 15 min linear (5–35%) acetonitrile gradient in 0.1% formic acid at 300 nl/min using an Eksigent Nano1D + LC (Eksigent, Dublin, CA, USA). The column was washed with 90% acetonitrile—0.1% formic acid for 5 min and then re-equilibrated with 5% acetonitrile—0.1% formic acid for 10 min. The Applied Biosystems 5600 TripleTOF mass spectrometer (AB-Sciex, Toronto, Canada) was used to analyze the protein digest. In-house MASCOT database searches were carried out against the Homo sapiens genome on the NCBI database. The mass tolerances for precursor scans and MS/MS scans were set at 0.05 Da. One missed cleavage for trypsin was allowed. A fixed modification of carbamidomethylation was set for cysteine residues; and a variable modification of oxidation was allowed for methionine residues. Proteins with at least one individual peptide MOWSE score of >40 were considered significant.

2.6. Subcellular fractionation

Subcellular fractionation was performed in parallel for each pair of subjects using nitrogen cavitation and differential sucrose gradient centrifugation as described previously (Hammond et al., 2012). This protocol yields endoplasmic reticulum (ER), nuclear, and mitochondrial (MT) enriched fractions. It also yields a fraction containing cytosolic (glutamine synthetase), endosomal (early endosomal antigen 1), and extrasynaptic membrane (EAAT2 and GluA4) markers; this “extrasynaptic membrane/cytosol” fraction (referred to as the ES fraction hereafter) does not contain synaptic (such as PSD95) markers (Hammond et al., 2012). The homogenate from each of 16 paired DLPFC subjects was centrifuged at 700 g, 4 °C for 10 min. A subsequent 15,000 g centrifugation pelleted mitochondria, the pellet was then resuspended in 500 μl of 1× PBS as a mitochondrial fraction (MT fraction). In a 14× 89-mm Beckman polyallomer ultracentrifuge tube, 1.3 M (1 ml), 1.5 M (1 ml) and 2.0 M (1 ml) sucrose solutions were sequentially layered. The resulting supernatant was layered onto the gradient and then ultracentrifuged at 126,000 g (35,000 rpm in an SW60 Ti rotor) at 4 °C for 70 min. The upper 200 μl of solution from the tube was withdrawn and labeled as a fraction containing extrasynaptic membranes and cytosol (ES fraction). 100 μl to 300 μl of a dense band at the interface of the 1.3 M sucrose

gradient layer were extracted, and combined with cold $1 \times$ MTE (270 mM D-mannitol, 10 mM Tris-base, 0.1 mM EDTA, pH 7.4) buffer plus phenylmethylsulfonyl fluoride (PMSF, 1 mM) to dilute out the sucrose. The ER pellet was obtained by ultracentrifugation of this fraction at 126,000 g (35,000 rpm in an SW60 Ti rotor), 4 °C for 45 min and then resuspended in 50 μ l of $1 \times$ PBS, pH 7.4 as ER enriched fraction (ER fraction). The protein concentration for all fractions was analyzed by a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

2.7. Electron microscopy

Samples were prepared for electron microscopy as previously described (Hammond et al., 2012). Briefly, ES and MT fractions were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at room temperature, and then washed and treated with 1% osmium tetroxide for 1 h, mordanted with 0.25% uranyl acetate in acetate buffer for 30 min to overnight, washed and dehydrated with a graded series of ethanol washes and propylene oxide. Finally, the samples were embedded in epoxy resin, thin sectioned and counterstained with uranyl acetate and lead citrate. Images were captured using an FEI Tecnai Spirit 20–120 kV Transmission Electron Microscope.

2.8. Western blot analysis

Due to limited amounts of material from our fractionation experiment, we were only able to run 13 pairs of subjects for the EAAT2 exon 9 skipping and 12 pairs for the Na^+/K^+ ATPase β studies. Samples for western blot analyses were prepared with milli-Q water and sample buffer ($6 \times$ solution: 4.5% sodium dodecyl sulfate (SDS), 15% β -mercaptoethanol, 0.018% bromophenol blue, and 36% glycerol in 170 mM Tris-HCl, pH 6.8) and heated at 70 °C for 10 min. For protein analysis of subcellular fractions, the same amount of protein (5–10 μ g) was loaded for each subject pair (Hammond et al., 2012). Samples were then run on 4–12% gradient gels and transferred to PVDF membranes by BioRad semi-dry transblotters (Bio-Rad, Hercules, CA, USA). The membranes were blocked with LiCor blocking buffer (LiCor, Lincoln, NE, USA) for all antibodies except EAAT2B which was blocked with 5% BSA for 1 h at room temperature, then probed with the primary antibodies. After three 8 min washes in $1 \times$ PBS, the membranes were then incubated with the appropriate second antibody with IR-Dye 670 or 800cw labeled in LiCor blocking buffer or 5% BSA in PBS for 1 h at room temperature. Washes were repeated after secondary antibody incubation. Membranes were scanned using a Li-Cor Odyssey scanner, and the intensity value for each protein band was measured using the Odyssey 2.1 software.

2.9. Data analysis

Mass spectrometry data was analyzed with software of Database for Annotation, Visualization and Integrated Discovery (DAVID). Proteins were identified as interacting proteins in the immunoprecipitation if they were present in the immunoprecipitation but not in the IgG control, or they were at least 2-fold higher in the immunoprecipitation compared to the paired IgG control. The 2-fold higher threshold was based on 1) immunoprecipitation studies with GLT1 (rodent EAAT2) that found statistically significant changes in protein–protein interactions (with GLT1) at the 2-fold change level (Sidoryk-Wegrzynowicz et al.,

2012), and 2) statistical methods developed for large datasets (such as transcript microarrays) that use the fold change approach (Beasley et al., 2005; Dalman et al., 2012). Proteins common to both EAAT2 and Na⁺/K⁺ ATPase immunisolations were considered part of the glutamate transport protein complex. Image analysis from Western blot studies was performed as previously described (Hammond et al., 2012). Briefly, near-infrared fluorescent signals obtained from the LiCor Odyssey scanner were expressed as integrated intensity with top-bottom median intralane background subtraction using Odyssey 3.0 analytical software (LiCor, Lincoln, NE, USA). For Western blot analysis of subcellular fractions, band intensity for each protein in the fractions was normalized to the protein band intensity for the same protein loaded in the total homogenate lane (Hammond et al., 2012). Paired t-tests were used to analyze the data using Graphpad Prism 3.0 (Graphpad Prism, La Jolla, CA, USA). Multiple regression analysis was performed to probe for possible correlations between protein expression and age, postmortem interval, and/or tissue pH (Statsoft, Tulsa, Oklahoma, USA). Analysis of variance (ANOVA) was also performed to assess the effects of sex and antipsychotic status on the dependent measures. For antipsychotic status, subjects were considered off antipsychotics if antipsychotics were not administered within 6 weeks of death (Statsoft, Tulsa, Oklahoma, USA).

3. Results

3.1. Characterization of EAAT2B and EAAT2 exon 9 skipping antibodies

The EAAT2B and EAAT2 exon 9 skipping antibodies were designed based on published sequences used to generate similar antibodies (Maragakis et al., 2004; Macnab and Pow, 2007) and detected bands at the predicted molecular weights of 73 kD (Fig. S1, 1) or 50 kD (Fig. S2, 1) on Western blots, respectively. We also performed blocking peptide experiments with these antibodies (Fig. S1 and S2). These bands were selectively diminished by blocking peptides (Fig. S1, 2; Fig. S2, 2). The previously well-characterized EAAT2 antibody detected a band at 62 kD (Fig. S1, 3; Fig. S2, 3) (Bauer et al., 2010). As expected, blocking peptides for the EAAT2b (Fig. S1, 4) or EAAT2 exon 9 skipping antibodies (Fig. S2, 4) did not block immunoreactivity of EAAT2 antibody.

3.2. Identification of a glutamate transport protein complex in human postmortem prefrontal cortex

We performed immunoisolation (Fig. 1 and Supplementary Fig. S3) and mass spectrometry to identify proteins interacting with EAAT2 or Na⁺/K⁺ ATPase (Fig. 2, Tables 1–2). The identified proteins were grouped based on software of Database for Annotation, Visualization and Integrated Discovery (DAVID). Several proteins including EAAT2, Na⁺/K⁺ ATPase subunits, HK1 and aconitase subunits were identified in both of EAAT2 and Na⁺/K⁺ ATPase interactomes (Fig. 2, lower box) suggesting that EAAT2, Na⁺/K⁺ ATPase, HK1, and aconitase 1 and 2 comprise a glutamate transport protein complex in human postmortem prefrontal cortex.

3.3. Expression of glutamate transport protein complex constituents in DLPFC total homogenate

We measured levels of EAAT2 and Na⁺/K⁺ ATPase interacting proteins in total homogenates from paired control and schizophrenia subjects by Western blot. We did not detect any difference in EAAT2 (Fig. 3A), EAAT2B (Fig. 3B), EAAT2 exon 9 skipping (Fig. 3C, N = 13), Na⁺/K⁺ ATPase α 1 (Fig. 3D), pan Na⁺/K⁺ ATPase β (Fig. 3E), HK1 (Fig. 3F), aconitase 1 (Fig. 3G) or aconitase 2 (Fig. 3H) proteins in subjects with schizophrenia and comparison subjects.

No significant correlations were found between protein expression and tissue pH, postmortem interval or age. No significant differences were found in subjects with schizophrenia on antipsychotic medications at the time of death, compared to subjects off medications for at least 6 weeks (data not shown).

3.4. Assessment of extrasynaptic membrane and mitochondrial fractions

We have previously developed a technique to fractionate human postmortem brain tissue (Hammond et al., 2012). In the present study, we have further characterized this fractionation protocol. We assessed ES and MT fractions by Western blot and electron microscopy. Expression of voltage-dependent ion channel (VDAC), an outer mitochondrial membrane protein, was enriched in the MT fraction, but not in total homogenate (T), ER or ES fractions (Fig. 4A). As expected, the MT fraction contains an enrichment of intact mitochondria (Fig. 4B, right panel). We previously characterized the ES fraction as containing proteins localized to extrasynaptic membranes and cytosol (Hammond et al., 2012). We did not detect MT, nuclei or microsomal membranes in the ES fraction using electron microscopy (Fig. 4B, left).

3.5. Expression of glutamate transport protein complex constituents in subcellular fractions

In order to determine whether there are abnormalities of protein expression in subcellular fractions, we first examined the expression of EAAT2 and its splice variants including EAAT2B and EAAT2 exon 9 skipping proteins in fractions in the DLPFC of 16 pairs of subjects. We found that EAAT2B was significantly increased in ES fraction in subjects with schizophrenia (Fig. 5B, $P < 0.05$). No significant changes were found in the expression of EAAT2 (Fig. 5A) or EAAT2 exon 9 skipping (Fig. 5C) proteins in ER, ES or MT fractions in schizophrenia. Next, we examined the expression of glutamate transport complex proteins in ES and MT fractions. Aconitase 1 was significantly increased in MT fraction in subjects with schizophrenia (Fig. 6D, $P < 0.05$). No significant changes were found for Na⁺/K⁺ ATPase α 1 (Fig. 6A), Na⁺/K⁺ ATPase β (Fig. 6B), HK1 (Fig. 6C) or aconitase 2 (Fig. 6E) protein levels in subjects with schizophrenia.

No significant correlations were found between protein expression and tissue pH, postmortem interval or age. No significant differences were found in subjects with schizophrenia on antipsychotic medications at the time of death, compared to subjects off medications for at least 6 weeks (data not shown).

3.6. Partitioning of HK1, aconitase 1 and 2 in ES and MT fractions

We measured expression of HK1, aconitase 1 and 2 proteins in the ES, MT and ER fractions. To assess the partitioning of mitochondrial enzymes, we analyzed the ratio of each enzyme in ES/MT fractions in subjects with schizophrenia and comparison subjects. We found an increased ratio of HK1 in ES/MT in subjects with schizophrenia (Fig. 7, $P < 0.05$), suggesting a shift in expression from MT to cytosol. The ES/MT ratio of aconitase 1 or 2 was not significantly changed in the DLPFC (Fig. 7).

4. Discussion

In this study, we first identified a putative protein complex formed by EAAT2, Na^+/K^+ ATPase, HK1 and aconitase in human postmortem prefrontal cortex. This finding is based on the data that these proteins were detected in both EAAT2 and Na^+/K^+ ATPase interactomes using immunoisolation and mass spectrometry. We postulate that EAAT2, Na^+/K^+ ATPase, HK1 and aconitase form a protein complex on the membranes of astrocytic processes that rapidly removes glutamate from the perisynaptic space, maintains low basal levels of glutamate in the synaptic cleft, and limits the pool of glutamate available to spill out of the synapse.

Previous work has described an extensive link between glutamate transport and the Na^+/K^+ ATPase (Blanco and Mercer, 1998; Cholet et al., 2002; Kawakami and Ikeda, 2006; Rose et al., 2009; Larsson et al., 2010). In mouse cerebral astrocytes, increased glutamate uptake following Na^+ loading was inhibited by ouabain, a specific inhibitor of Na^+/K^+ ATPase (Pellerin and Magistretti, 1997). In synaptosomes isolated from forebrain and cerebellum, ouabain impairs GLT1/EAAT2 activity, and injection of ouabain induces excitotoxicity secondary to cellular membrane depolarization in neonatal rats (Genda et al., 2011). Mice defective in the Na^+/K^+ ATPase $\alpha 2$ subunit gene showed impaired reuptake of glutamate, enhanced neural excitation, and cell death in brain tissue (Ikeda et al., 2003; Kawakami and Ikeda, 2006; Moseley et al., 2007). Furthermore, colocalization of Na^+/K^+ ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ with GLT-1/EAAT2 was observed in the CA1 region of the rat hippocampus and rat cortex by immunocytochemical analyses, coimmunoprecipitation and mass spectrometry (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011). A similar relationship between Na^+/K^+ ATPase, glycolysis and mitochondria was also found in several cell culture studies (Parker and Hoffman, 1967; Balaban and Bader, 1984; Lynch and Balaban, 1987; Pellerin and Magistretti, 1994; Abu-Hamad et al., 2008). Activation of K^+ uptake into brain cells stimulated by small increases in extracellular K^+ is specifically dependent on glycolytically generated energy in hippocampal slices, an effect also abolished by ouabain (Lipton and Robacker, 1983). Finally, another study found that GLT1/EAAT2, Na^+/K^+ ATPase, HK1 and mitochondria are colocalized in rat astrocytic processes (Genda et al., 2011), consistent with our findings in human brain. Taken together, these findings suggest that there is a glutamate transport protein complex comprised of EAAT2, Na^+/K^+ ATPase and glycolytic enzymes that are linked to mitochondria and facilitate efficient reuptake of glutamate from the synaptic cleft into astrocytes.

EAAT2 is synthesized in the ER and undergoes extensive posttranslational modification in the Golgi, including N-linked glycosylation. It is then trafficked to the plasma membrane

where localization and clustering are regulated by protein–protein interactions and phosphorylation (Kalandadze et al., 2004). A number of studies have reported region-level changes in the expression of EAAT2 mRNA in schizophrenia. Decreased EAAT2 mRNA expression was found in the hippocampus; decreased, increased and no change in EAAT2 mRNA expression was found in the frontal cortex in three different studies (Ohnuma et al., 1998; Lauriat et al., 2006; Bauer et al., 2008; Rao et al., 2012). Previously, we found decreased EAAT2 protein levels in the hippocampus and superior temporal gyrus (STG) in subjects with schizophrenia (Shan et al., 2013). Although we detected no changes in EAAT2 protein levels in the prefrontal cortex, we did find an increase in G-protein pathway suppressor-1 (GPS1) protein and diminished EAAT2 glycosylation in the frontal cortex in schizophrenia (Bauer et al., 2008, 2010). Overall, these findings suggest abnormalities in trafficking of EAAT2 to the plasma membrane, raising the question of whether the distribution of EAAT2 or EAAT2 interacting proteins in subcellular fractions may be abnormal.

We previously developed a technique to fractionate human postmortem brain tissue (Hammond et al., 2012). This protocol was initially developed to assess the ER, and we verified that the ER fraction was enriched for ER markers by Western blot analysis and electron microscopy (Hammond et al., 2012). We have further refined this protocol to improve the enrichment of the other fractions from postmortem samples. In the present study, we optimized the ES and MT fractions. Our data demonstrate that the ES fraction does not contain mitochondria, nuclei, or microsomal membranes, while the MT fraction is enriched for mitochondria (Fig. 4).

To test the hypothesis that the subcellular distribution of EAAT2 may be abnormal, we measured expression of EAAT2 and EAAT2 splice variants in ER, ES and MT fractions in schizophrenia. While EAAT2 is primarily expressed perisynaptically on astroglial processes, expression of the EAAT2B variant is more diffuse in the cytoplasm of astrocytes and neurons (Maragakis et al., 2004; Lauriat and McInnes, 2007). Consistent with these findings, we detected EAAT2 and EAAT2B proteins in the ES fraction, which includes cytoplasm and extrasynaptic membranes from these cell types. Interestingly, we found that EAAT2B and EAAT2 exon 9 skipping proteins have a high ES fraction selectivity (Fig. 5), whereas EAAT2 protein has high levels of expression in the ER and ES fractions (compared to the splice variants). We hypothesize that there is a larger fraction of native EAAT2 expressed in the ER compared to the other splice variants. Similar to other proteins, such as the AMPA receptor subunit GluA2, there may be a regulatable pool of EAAT2 in the ER, which may be rapidly mobilized to the plasma membrane when increased glutamate reuptake or buffering is required.

Although EAAT2B has 11 unique amino acids at the C-terminus compared to native EAAT2, it retains the ability to transport glutamate, and likely regulates extrasynaptic glutamate levels (Maragakis et al., 2004; Lauriat and McInnes, 2007). In amyotrophic lateral sclerosis, EAAT2B protein was significantly increased in postmortem motor cortical neurons compared with controls (Maragakis et al., 2004). Although one study examined EAAT2B transcripts and found no difference in DLPFC in schizophrenia (Lauriat et al., 2006), we have found increased EAAT2B transcripts in thalamic relay neurons in this illness

(unpublished observation), suggesting an increase in neuronal expression of EAAT2B under pathological conditions. In the present study, we found increased EAAT2B protein in the ES fraction in schizophrenia, suggesting that increased EAAT2B may have a compensatory role by contributing to the regulation of glutamate levels in extrasynaptic regions in response to impaired astrocytic reuptake of glutamate. In contrast, we didn't find changes in EAAT2 exon 9 skipping variant, suggesting that changes in EAAT2 splice variant expression are specific to EAAT2B.

Another putative constituent of the glutamate transporter protein complex, aconitase, has two isoforms. Aconitase 1 is primarily localized in cytoplasm, serving as both an enzyme and an iron regulatory protein (IRP), while aconitase 2 is localized to mitochondria, converting citrate to isocitrate via a *cis*-aconitate in the second step of the tricarboxylic acid (TCA) cycle. In this study, we found that aconitase 1 was primarily expressed in the ES fraction (which contains cytosol), while aconitase 2 was expressed in both the ES and MT fractions. One alternative explanation for the presence of aconitase 1 and 2 in ES fraction is that they may be associated with extrasynaptic membrane-bound protein complexes that segregate together in our fractionation protocol (while the mitochondria are stripped away). Association of aconitase 1, generally considered a cytosolic enzyme, with a transmembrane protein complex is rather surprising and may indicate a novel protein interactome. These associations may be physically occurring via matrix or intermediary proteins like those described for HK1 (Genda et al., 2011). Another possibility is that some of the mitochondria in our samples may be compromised, and have leaked their contents during the postmortem interval or after a freeze thaw cycle. Such an effect could increase the variability of our dependent measures. For example, the apparently large increases for aconitase 1 in the ES fraction (Fig. 6D) and aconitase 2 in the ES and MT fractions (Fig. 6E) are non-significant, likely due to higher variance in these particular experiments. Finally, higher variability in the schizophrenia group might reflect an endogenous feature of this substrate, as subjects with schizophrenia generally have higher statistical variability than control subjects (MacDonald et al., 2006).

Aconitase is a biomarker of mitochondrial and cellular oxidative stress. During oxidative stress, aconitase activity is inhibited, leading to a decrease in energy production. In nerve terminals isolated from brain cortex of guinea pigs, aconitase inactivation blocks NADH production, and the TCA cycle is blocked when aconitase is completely inactivated (Tretter and Adam-Vizi, 2000). One group found decreased aconitase activity in DLPFC in schizophrenia (Bubber et al., 2011). Our finding of increased aconitase 1 in MT fraction may reflect a compensatory response to reduced activity of aconitase enzymes or a decrease in coupling of hexokinase activity and mitochondrial oxidative phosphorylation.

The immediate energy source for the active transport of Na^+ and K^+ is provided by the hydrolysis of ATP. A number of studies have demonstrated that glutamate released from excitatory synapses stimulates glycolysis in astrocytes; capacity for transport via the Na^+/K^+ ATPase is greater when ATP is produced from both glycolysis and oxidative phosphorylation than when ATP is produced from oxidative phosphorylation alone (Lynch and Balaban, 1987; Pellerin and Magistretti, 1994; Magistretti and Pellerin, 1999; Magistretti et al., 1999; Rothman et al., 1999). HK1 is an initial enzyme in glycolysis that

catalyzes the phosphorylation of glucose to form glucose 6-phosphate (Wilson, 2003). HK1 is expressed abundantly in the brain and typically localizes to the outer membrane of mitochondria (Wilson, 2003; Abu-Hamad et al., 2008). Interestingly, recent evidence shows that genes that regulate glucose metabolism may also influence susceptibility to schizophrenia (Stone et al., 2004). One postmortem study found a decrease in HK1 attachment to the outer membrane of mitochondria in parietal cortex in schizophrenia (Regenold et al., 2012). We found a large increase in the ratio of HK1, but not aconitase 1 or 2 in the ES/MT fractions in schizophrenia. These data suggest that the integrity of the glutamate transport protein complex may be disrupted in this illness. One possibility is that the whole complex is no longer associated with mitochondria. Another possibility is that HK1 localization is selectively altered, with a shift of this protein to the cytosol. Regardless of the mechanism, dissociation of HK1 from MT might decrease ATP production, decreasing the efficiency of the Na^+/K^+ ATPase, leading to impaired glutamate reuptake.

Na^+/K^+ ATPase is a plasma membrane protein comprised of α and β subunits. The α subunit contains the binding sites for the cations, ATP, and the inhibitor ouabain; the β subunit stabilizes the correct folding of the α to facilitate its delivery to the plasma membrane (McDonough et al., 1990; Blanco and Mercer, 1998). Our data shows that the $\alpha 1$ subunit is largely expressed in the MT fraction. This result was unexpected as $\alpha 1$ is reported to be primarily expressed in extrasynaptic membranes and not associated with mitochondria (Hundal et al., 1994; Blanco and Mercer, 1998). Taken together with our immunoisolation data, we propose that the $\alpha 1$ subunit is expressed with the glutamate transport protein complex affiliated with MT in astrocytes. In the present study, we have focused on $\alpha 1$ and β subunits due to their broad tissue and cell distribution (Cameron et al., 1994; Blanco and Mercer, 1998), as well as demonstration of colocalization of $\alpha 1$ or $\beta 1$ with GLT1/EAAT2 and mitochondria in rat brain cortex (Genda et al., 2011). While we didn't find significant changes in Na^+/K^+ ATPase $\alpha 1$ and β proteins in total homogenate or subcellular fractions, there was about a 4-fold increase (non-significant; $p = 0.13$) in Na^+/K^+ ATPase β protein in the ES fraction (Fig. 6B) suggesting that there may be fraction-specific changes in this protein in schizophrenia.

There are several potential limitations of this study. Our secondary analyses found no differences in expression of the complex proteins between medicated patients and patients who had been free of anti-psychotic medications for at least 6 weeks prior to death. However, the possibility remains that our findings might be impacted by the subjects taking (mostly) typical antipsychotic medications, as being off medication for 6 weeks may not reverse a lifetime of neuroplastic changes secondary to decades of antipsychotic treatment (Schneider et al., 1998; De Souza et al., 1999; Melone et al., 2001). Another concern is that our cohort was aged, and changes found in the later stages of the illness may not reflect the pathophysiology of the illness at the early or mid-stage (Clinton et al., 2003; Clinton and Meador-Woodruff, 2004). However, a strength of this aged cohort is that most of the subjects were institutionalized, did not have access to alcohol or drugs of abuse, and tended to die of natural causes, rather than drug abuse or suicide.

In this study we have generated two antibodies (EAAT2B and EAAT2x9) using sequences previously employed to successfully generate specific antibodies for these EAAT2 splice

variants (Maragakis et al., 2004; Lauriat and McInnes, 2007; Macnab and Pow, 2007). Our antibodies do not detect their targets in rodent tissues (unpublished observation), making the use of EAAT2 KO tissues to characterize these antibodies unfeasible. However, we did detect bands at the predicted relative migration distance for each variant, and these bands were selectively diminished with blocking peptide. Since we have not definitely characterized these new antibodies (for example with human cell lines expressing the variants), the possibility remains that our EAAT2B and EAAT2x9 antibodies may not be specific. In contrast, the pan-EAAT2 antibodies used for the immunoisolation and Western blots are previously well-characterized with regard to specificity, indicating that our results identifying EAAT2 interacting proteins do not share this limitation (Ginsberg et al., 1995; Bar-Peled et al., 1997; Ikematsu et al., 2001; Bjornsen et al., 2007).

While our results show the enrichment of ER, ES and MT fractions verified by Western blots and electron microscopy, the possibility remains of cross-contamination between fractions due to freeze-thaw effects unavoidable in human postmortem brain samples. The nature of this protocol makes it difficult to ascertain percent purity/enrichment of the fractions. This stems from the problem that biological markers for each of the fractions may be found at low levels in other, non-analyzed fractions. Our studies used cortical brain homogenate as a starting point. We then fractionated these samples, but brain homogenates are an admixture of multiple cell types and specialized structures, including axons, dendrites, and white matter tracks. It is possible, for example, that changes in protein expression or localization could be increased in one cell type, but decreased in another, yielding no net change in the dependent measure.

In summary, we postulate that a glutamate transport protein complex is formed by EAAT2, Na⁺/K⁺ ATPase, HK1 and aconitase proteins in human postmortem prefrontal cortex. Changes in EAAT2B, aconitase 1, and HK1 expression suggest that the composition and subcellular localization of complex proteins may be abnormal in schizophrenia (Fig. 8). A decrease in glutamate reuptake capacity could lead to glutamate spillover from excitatory glutamate synapses, activating extrasynaptic glutamate receptors or receptors in adjacent synapses. Such changes in the structure and function of glutamate circuits could alter synaptic plasticity, contributing to the cognitive deficits attributed to frontal cortical dysfunction in this illness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Immunoisolation of EAAT2 and Na^+/K^+ ATPase from human postmortem prefrontal cortex.

A) Western blot (WB) of EAAT2 immunoprecipitated with rabbit anti-EAAT2 antibody. The black arrows indicate a monomer (62KD), dimer (120KD) and trimer (200KD) of EAAT2.

B) Western blot of Na^+/K^+ ATPase $\alpha 1$ immunoprecipitated with rabbit pan anti- Na^+/K^+ ATPase antibody (black arrow). Prefrontal cortical lysates (600 μg protein) prepared with 1% SDS were used for the immunoisolation. 10 μg of lysate (Lys) was loaded as a positive control, Rabbit IgG (IgG) was used as a negative control in the immunoisolation.

Abbreviations: Immunoisolation (II), Western blot (WB), excitatory amino acid transporter (EAAT), tissue lysate (Lys), Na^+/K^+ ATPase $\alpha 1$ (NaKA $\alpha 1$).

Fig. 2. Identification of glutamate transport protein complex in human postmortem prefrontal cortex. Identified proteins were grouped based on software of Database for Annotation, Visualization and Integrated Discovery (DAVID). The left box shows grouped proteins identified from immunoaffinity purified EAAT2 (EAAT2 interactome). The right box shows grouped proteins identified from immunoaffinity purified Na⁺/K⁺ ATPase (Na⁺/K⁺ ATPase interactome). The lower box shows proteins common to the EAAT2 and Na⁺/K⁺ ATPase interactomes.

Fig. 3.

Western blot analysis of glutamate transport protein complex in DLPFC total homogenate in paired schizophrenia and comparison subjects. Data are expressed as mean $\pm$ standard error. Expression of EAAT2 (A), EAAT2B (B), EAAT2 exon 9 skipping (C), Na⁺/K⁺ ATPase $\alpha 1$ (NaKA $\alpha 1$, D), Na⁺/K⁺ ATPase β (NaKA β , E), Hexokinase1 (HK1, F), aconitase 1 (G) and aconitase 2 (H) proteins in total homogenate was not significantly changed in subjects with schizophrenia. Schizophrenia (SCZ); control (CTL).

Fig. 4. Assessment of extrasynaptic membrane and mitochondrial fractions isolated from human postmortem prefrontal cortex by Western blot and electron microscopy. A) Western blot shows enriched expression of the mitochondrial marker, voltage-dependent ion channel (VDAC), in the mitochondrial fraction (MT). VDAC was not detected in the extrasynaptic membrane (ES) fraction. The black arrow indicates the VDAC band on the Western blot. B) Electron microscope image (left, 4400 $\times$) showing that the extrasynaptic membrane fraction contains structures resembling light membranes not contaminated with other subcellular organelles. Electron microscope image (right, 4400 $\times$) showing an enrichment of intact mitochondria in the mitochondrial fraction (red arrows and inset).

Fig. 5.

Western blot analysis of EAAT2 and EAAT2 splice variants in subcellular fractions. Data are reported as mean $\pm$ standard error of the values generated for each subject expressed as relative expression in the fraction normalized to total homogenate within each assay. EAAT2B (B), a splice variant of EAAT2, was significantly increased in ES fraction in subjects with schizophrenia. No significant changes were observed for EAAT2 (A) or EAAT2 exon 9 skipping (C). Representative Western blot images shown on the left for one subject pair. * $P < 0.05$.

Fig. 6.

Western blot analysis of Na⁺/K⁺ ATPase subunits, HK1 and aconitase subunits in extrasynaptic membrane/cytosol (ES) and mitochondrial (MT) fractions. Data are reported as mean $\pm$ standard error of the values generated for each subject expressed as relative expression in the fraction normalized to total homogenate within each assay. Expression of aconitase 1 protein (D) was significantly increased in MT fraction in subjects with schizophrenia. No significant changes were found in the expression of Na⁺/K⁺ ATPase α 1 (A), β (B), HK1(C) or aconitase 2 (E) proteins in subjects with schizophrenia. Representative Western blot images shown on the left for one subject pair. Arrows indicate the bands of interest. *P < 0.05.

Fig. 7.

Partitioning of HK1, aconitase 1 and 2 in extrasynaptic membrane/cytosol (ES) and mitochondrial (MT) fractions. Data expressed as the mean $\pm$ standard error of the ratios of the ES to MT fractions for each subject. A significantly increased ratio of HK1 in the ES/MT fractions was found in the DLPFC subjects with schizophrenia. The ES/MT ratio for aconitase 1 and 2 was not significantly changed in subjects with schizophrenia. * $P < 0.05$.

Fig. 8.

We characterized a protein complex containing EAAT2, Na⁺/K⁺ATPase, hexokinase 1 (HK1), aconitase 1 (AC1), and aconitase 2 (AC2) in human prefrontal cortex. In subjects with schizophrenia, expression of the EAAT2 splice variant EAAT2B (E2B) was significantly increased in a fraction containing extrasynaptic membranes and cytosol (ES), while expression of aconitase 1 was significantly increased in the mitochondrial fraction (MT). An increased ratio of hexokinase 1 protein expression in ES/MT fraction was also found in subjects with schizophrenia.

Table 1

Proteins identified by mass spectrometry in EAAT2 immunoisolation.

6-Phosphofructokinase type C isoform 1
Aconitase
Aconitate hydratase, mitochondrial precursor
Alpha II spectrin
Aminopeptidase
ATPase Na ⁺ /K ⁺ transporting alpha 4
Brain glycogen phosphorylase
C3b
Calmodulin-dependent protein kinase II
Centromere protein P
Chaperonin (HSP60)
Clathrin heavy chain 1
Dihydropyrimidinase-related protein 1 isoform 2
Dihydropyrimidinase-related protein 2 isoform 1
Dihydropyrimidinase-related protein 2 isoform 2
DPYSL3 protein
Dynamin
Endoplasmin precursor
Excitatory amino acid transporter 2
Fascin
Ga subunit
Glial fibrillary acidic protein
Glutamate dehydrogenase (NAD(P)+)
Guanine deaminase isoform b
Guanine nucleotide-binding protein alpha-i
Guanine nucleotide-binding protein G(t) subunit alpha-3
hCG1983510, isoform CRA_a
Hexokinase type I
HSP90AB1 protein
KIAA0417
KIAA0607 protein
KIAA0719 protein
KIAA0968 protein
Motor protein
MTHSP75
Myelin proteolipid protein isoform 2
Na ⁺ ,K ⁺ -ATPase catalytic subunit
Na ⁺ /K ⁺ transporting ATPase alpha 3 polypeptide
NADH dehydrogenase (ubiquinone) Fe-S protein 1
Neural cell adhesion molecule (clone 4.4)

Neural cell adhesion molecule 1, isoform CRA_a
Neurofilament
Neuropilin-1 B1
NF-M
Nicotinamide nucleotide transhydrogenase
Non-gastric H⁺,K⁺-ATPase
Phosphofructokinase, liver
Potassium-transporting ATPase alpha
Rab GDP dissociation inhibitor alpha
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Serine/threonine-protein phosphatase 2B catalytic subunit
Sodium/potassium-transporting ATPase subunit alpha-1
Sodium/potassium-transporting ATPase subunit alpha-2
Synapsin I
Synapsin-1 isoform Ib
Synaptobrevin
Synaptotagmin-1
Syntaxin-binding protein 1
Transitional endoplasmic reticulum ATPase
Tumor necrosis factor type 1 receptor associated protein
Ubiquitin activating enzyme E1
Vacuolar-type H(+)-ATPase 115 kDa subunit

Table 2Proteins identified by mass spectrometry in Na⁺/K⁺ ATPase immunoisolation.

Alpha II spectrin
2',3'-Cyclic-nucleotide 3'-phosphodiesterase
2-Oxoglutarate dehydrogenase precursor
60 kDa heat shock protein, mitochondrial
90 kDa heat shock protein
Acetyl-coenzyme A acyltransferase 2
Aconitate hydratase, mitochondrial precursor
Adaptor-related protein complex 1, beta 1 subunit
Adenosine triphosphatase
Adenylyl cyclase-associated protein 1
Alanyl-tRNA synthetase
Alpha-actinin-2
Alpha-enolase isoform 1
Aminopeptidase
Ankyrin-3 isoform 1
Anti-pneumococcal antibody 3H1 light chain
AP3B2 protein
ATPase, Na ⁺ /K ⁺ transporting, alpha 2
ATP-specific succinyl-CoA synthetase beta subunit
Axonal transporter of synaptic vesicles
Bassoon protein
Beta-NAP
Brain acid soluble protein 1
Bullous pemphigoid antigen 1 isoform 1eA precursor
Cadherin-associated protein-related
Calcium/calmodulin-dependent protein kinase II beta
CaM kinase II gamma J
CAMK2D protein
Catenin beta-1
CDC10 homolog
cGMP-dependent 3',5'-cyclic phosphodiesterase isoform = 1
Clathrin heavy chain 1
Coatomer protein
Coatomer subunit beta
Coronin-like protein
Creatine kinase-B
CTNNA2 protein
Cytoplasmic dynein 1 heavy chain 1
Cytoplasmic dynein 1 intermediate chain 1 isoform a
DEAD box protein

Dermcidin preproprotein
De-ubiquitinase
Disks large homolog 4 isoform 1 precursor
DNA dependent protein kinase catalytic subunit
Dynactin
Dynamin
Dynamin 1
Dynamin-2 isoform 2
Dynamin-like 120 kDa protein, mitochondrial isoform 1
Elongation factor 2
Elongation factor Tu
Elongation factor-1 alpha
Encodes region of fatty acid synthase activity
Endoplasmin precursor
Erythroid K:Cl cotransporter splicing isoform 2
Excitatory amino acid transporter 2
Factor VII active site mutant immunoconjugate
Fatty acid synthase
Fructose-bisphosphate aldolase C
Glial fibrillary acidic protein isoform 1
Glutaminase
Glutaminase isoform C
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 isoform 1
H, K-ATPase catalytic subunit
hCG1790904, isoform CRA_b
hCG28233, isoform CRA_a
Heat shock 90 kDa protein 1, alpha
Heat shock protein HSP 90-alpha 2
Heavy neurofilament subunit
Hexokinase 1
hnRNP U protein
Internexin neuronal intermediate filament protein
Isocitrate dehydrogenase (NADP+)
Karyopherin beta 3
KIAA0002
KIAA0654 protein
KIAA0727 protein
KIAA0987 protein
Kinesin
Kinesin heavy chain isoform 5C
Lys48-linked tetraubiquitin
Macrophin 1 isoform 4
Major vault protein

Matrin 3
Matrin-3 isoform α
Microtubule-actin cross-linking
Microtubule-associated protein 1a
Microtubule-associated protein 2
Mitochondrial ATP synthase
MOBP
M-phase phosphoprotein 4
Myelin-associated glycoprotein isoform a precursor
MYH10 variant protein
Myosin
Na, K-ATPase beta subunit
Na⁺,K⁺-ATPase catalytic subunit
Na⁺,K⁺ ATPase
NADH:ubiquinone oxidoreductase 51-kD subunit
NEFL protein
Neural cell adhesion molecule CD56
Neurofascin homolog (chicken)
Neurone-specific enolase
NF-L
Nuclear dual-specificity phosphatase
Nuclear pore complex protein Nup155 isoform 2
Paraplegin-like protein
Phosphofructokinase
Phosphofructokinase, platelet
Placenta immunoregulatory factor PLIF
Plasma membrane calcium ATPase isoform 2
Plectin
Plectin 1
Plectin isoform 1
Plectin isoform 1e
Probable Xaa-Pro aminopeptidase 3 isoform 1
Proteasome subunit p112
Protein disulfide isomerase
Putative heat shock protein HSP90 beta-3
Putative heat shock protein HSP90-alpha A5
Putative hexokinase HKDC1
Putative tyrosine-protein phosphatase auxilin
Pyruvate carboxylase
Pyruvate kinase isozymes M1/M2 isoform b
Sarcolectin
SET binding factor 1, isoform CRA_c
SNAP-25-interacting protein

Sodium/potassium-transporting ATPase subunit alpha-1
Solute carrier family 12 member 6 isoform b
Spectrin, beta
Spectrin, beta, erythrocytic
Spermatid perinuclear RNA-binding protein
Spliceosome RNA helicase DDX39B
Splicing factor, proline- and glutamine-rich
Synaptosomal-associated protein 25
Syntaxin-1B
Syntaxin-binding protein 1
Talin 2
T-complex protein 1 subunit beta isoform 1
Tenascin R
Tetrahydrofolate synthase
Tumor necrosis factor type 1 receptor associated protein
Ubiquitin B, isoform CRA_d
Ubiquitin-like modifier-activating enzyme 1
Vacuolar-type H(+)-ATPase 115 kDa subunit
WDR1 protein