Neurochemistry International 125 (2019) 111–116

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

GLAST (GLutamate and ASpartate Transporter) in human prefrontal cortex; interactome in healthy brains and the expression of GLAST in brains of chronic alcoholics

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ARTICLE INFO

Keywords:
Alcohol
Prefrontal cortex
Human brain
Glutamate transport
Interactome
Proteomics

ABSTRACT

We have analysed post-mortem samples of prefrontal cortex from control and alcoholic human brains by the technique of Western blotting to estimate and compare the expressions of glutamate transporter GLAST (Excitatory Amino Acid Transporter One; EAAT1). Furthermore, using the non-alcoholic prefrontal cortex and custom-made GLAST (EAAT1) antibody we determined GLAST (EAAT1) "interactome" i.e. the set of proteins selectively bound by GLAST (EAAT1). We found that GLAST (EAAT1) was significantly more abundant (about 1.6-fold) in the cortical tissue from alcoholic brains compared to that from non-alcoholic controls. The greatest increase in the level of GLAST (EAAT1) was found in plasma membrane fraction (2.2-fold). Additionally, using the prefrontal cortical tissue from control brains, we identified 38 proteins specifically interacting with GLAST (EAAT1). These can be classified as contributing to the cell structure (6 proteins; 16%), energy and general metabolism (18 proteins; 47%), neurotransmitter metabolism (three proteins; 8%), signalling (6 proteins: 16%), neurotransmitter storage/release at synapses (three proteins; 8%) and calcium buffering (two proteins; 5%). We discuss possible consequences of the increased expression of GLAST (EAAT1) in alcoholic brain tissue and whether or how this could disturb the function of the proteins potentially interacting with GLAST (EAAT1) in vivo. The data represent an extension of our previous proteomic and metabolomic studies of human alcoholism revealing another aspect of the complexity of changes imposed on brain by chronic long-term consumption of ethanol.

1. Introduction

L-Glutamate (glutamate) is the most important excitatory neurotransmitter of the central nervous system (CNS); (Fonnum, 1984; Bennett and Balcar, 1999; Danbolt, 2014). Excessive extracellular concentrations of glutamate are, however, potentially neurotoxic and could be involved in etiology of many diseases from neuroinflammatory to neurodegenerative (reviews: Sheldon and Robinson, 2007; Getts et al., 2008; Lewerenz and Maher, 2015). Glutamate is removed by rapid transport mediated by specialized protein molecules (glutamate transporters) residing in plasma membranes, mostly in cells surrounding glutamatergic synapses. Five subtypes of glutamate transporters have been identified and named EAAT1 – EAAT5 (products of genes *SLC1A3, SLC1A2, SLC1A1, SLC1A6, SLC1A7*). The subtypes EAAT1 and EAAT2 are located in glial cells (astrocytes, microglia and oligodendrocytes), however, a variant of EAAT2 has also been found in axon-terminals (Danbolt et al., 2016). EAAT2 is responsible for over 90% of glutamate reuptake within the central nervous system (CNS) (reviews: Zhou and Danbolt, 2013; Danbolt et al., 2016). EAAT3 and EAAT4 are present in neurons and have variously been reported as expressed in axon terminals, cell bodies, and dendrites (Danbolt, 2001; Danbolt et al., 2016). EAAT5 is located mainly in the retina but could have much wider distribution both elsewhere in the CNS and in the periphery (Danbolt, 2001; Lee et al., 2012, 2013; 2016). The nomenclature based on EAAT ("excitatory amino acid transporter") is used mainly for the glutamate transporters isolated from human or guinea pig brain tissue; in rodents EAAT1 is referred to as GLAST; EAAT2 is called GLT1 and EAAT3 is known as EAAC3 (reviews: Danbolt, 2001; Balcar, 2002; Šerý et al., 2015).

Repeated exposure to ethanol has been shown to elevate extracellular glutamate levels while reducing glutamate uptake without changing the expression of GLAST (EAAT1) in the nucleus accumbens

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https://doi.org/10.1016/j.neuint.2019.02.009
Received 12 November 2018; Received in revised form 5 February 2019; Accepted 16 February 2019
Available online 25 February 2019
0197-0186/

(NAc); (Melendez et al., 2005). In contrast, using human brain post-mortem tissue, Flatscher-Bader and Wilce (2008) found dramatic increases in EAAT1 expression in deep layers of the prefrontal cortex whilst Rimondini et al. (2002) reported that chronic intermittent ethanol self-administration by rats induced GLAST (EAAT1) gene expression in the frontal cortex 5.7-fold. Experiments with GLAST knockout mice, however, indicated that GLAST −/− animals had lower alcohol consumption with no impact on ethanol preference in the conditioned place preference (CPP) paradigm (Karlsson et al., 2012). There is no easy and straightforward interpretation of the above findings. The data may have been influenced by species differences, by brain regional variations in the sensitivity to ethanol, by the differences in the modes of ethanol administration as well as by actual doses (particularly difficult to determine accurately when using human post-mortem tissue) and the length of time for which it was given. The above data, nevertheless, point to GLAST as potentially one of the key proteins involved in the response of brain tissue to ethanol, particularly when ethanol is administered repeatedly for a long time. As such, GLAST (EAAT1) should be considered a molecule of interest when investigating mechanisms of alcoholism.

There is evidence that glutamate transport acts in a close association with other proteins, particularly in conjunction with Na^+,K^+-dependent ATPase that produces Na^+ and K^+ gradients which provide the main driving force for the glutamate transport (Pellerin and Magistretti, 1986; Nanitsos et al., 2004). GLAST (EAAT1) in particular has been shown to bind and, possibly, cluster with a number of proteins, probably forming a complex ("transportosome") thus perhaps facilitating its normal functioning (Bauer et al., 2012).

The aims of the present study are twofold: first, we have estimated, using custom-made GLAST-specific antibodies and the technique of Western blotting, changes in the expression of GLAST (EAAT1) in human alcoholic brains and, second, using the same antibody and proteomic approach, we determined the "interactome" of GLAST (EAAT1) in human brain tissue. The data provide a broad database listing the proteins potentially interacting with GLAST (EAAT1) in human brain cells and indicate which additional molecules (apart from GLAST itself) may, therefore, be affected in human alcoholic brains over-expressing GLAST (EAAT1) transporter.

2. Materials and methods

2.1. Human brain tissue

Post-mortem human brains were obtained from the NSW Brain Bank at The University of Sydney. Six alcoholic brains and six controls were used. All donors were males. Control brains were from subjects aged 37–74 years (mean ± SEM: 59 ± 6) while the alcoholic brains came from patients aged 41–73 years (mean ± SEM: 59 ± 5). The causes of death were heart and circulation diseases (five cases) and respiratory arrest (one case) in controls; in the alcoholics the causes of death were heart and circulation diseases (four), epilepsy with chronic alcoholism (one) and respiratory arrest (one). The smoking status of the subjects was also recorded. There were smokers, non-smokers and ex-smokers in both groups (Table 1).

Even though the samples were matched as much as possible for age, gender, tissue pH and cause of death, in some parameters such as the post-mortem delay (PMI, post-mortem interval; Table 1) the values extended over a wide range, in both controls and alcoholic brains. The difference between the two samples was, however, not statistically significant ($P = 0.54$ by Student's t-test) and the variations in PMI had no apparent effect on the quality of data. Previous studies of proteins in human alcoholic brains suggested that changes in protein levels with PMI, at least within the range we used, were minimal and could be neglected (Lewohl et al., 2001 and references cited thereof). Indirect evidence using parameters such as the stability of nucleic acids in post-mortem human tissue (Trabzuni et al., 2011; Robinson et al., 2016) is consistent with that view.

Samples of prefrontal cortex (Teffer and Semendeferi, 2012) were collected and grouped as (i) control (< 20 g of ethanol/day) and (ii) alcoholics (> 80 g of ethanol/day). Post-mortem examination of the alcoholic brains revealed that there were no apparent complications from Wernicke–Korsakoff Syndrome (WKS) in any of the alcoholic brains used. Alcoholic cases fulfilled the criteria of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) for alcohol dependence (Anonymous, 1994); the relevant data were obtained from medical records or from reports by the next of kin (Kashem et al., 2016). Ethics approval for the study was granted by the Sydney South West Area Health Service (Protocol no. X13-0379).

2.2. Protein extraction

Protein extractions were performed according to the method previously described by Kashem and colleagues (Kashem et al., 2007, 2008, 2009a, 2009b; Hargreaves et al., 2009; Tang et al., 2009; van Nieuwenhuijzen et al., 2010). For cellular fraction collection, we have used sequential centrifugation technique. Post-mortem brain tissue was homogenized with CHAPS, $MgCl_2$ (1 mg) and 0.5% sodium deoxycholate buffer. The mixture was centrifuged at 3000 g for 15 min at 4 °C (nuclear fraction). The supernatant was further centrifuged at 100,000 g for 120 min at 4 °C. The pellet was re-suspended in sodium deoxycholate buffer (microsomal fraction; also contained plasma membranes). The supernatant was used as cytoplasmic fraction. For immunoprecipitation, the cortical tissues were extracted using urea buffer (7 M urea, 2 M thiourea and 1% C7bZO, 40 mM tris-HCl). The tissue extract was pelleted at 16,000 g for 20 min at 15 °C. The supernatant was reduced and exposed to 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 h. This was followed by dithiothreitol (DTT) to 10 mM to quench the reaction. The samples were subsequently acidified by citric acid to approximately pH 5. The acetone-precipitated pellet was dried and resuspended in buffer consisting of 7 M urea, 2 M thiourea and 1% C7bZO. Protein quantification was done according to Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

2.3. Western blot (WB) analysis

The extracted protein was separated by SDS-PAGE as described earlier (Kashem et al., 2016). The proteins were transferred to PVDF (polyvinylidene difluoride) membranes according to the protocol of Proteome IQ blotting kit (Proteome Systems Ltd, Australia) as described previously (Kashem et al., 2012). The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS; pH 7.4) and incubated with primary antibodies (GLAST, Shin et al., 2009) in the same blocking solution. Secondary antibodies (anti-rabbit IgG (Sigma)) were added according to the manufacturer's instructions and the resulting spots were visualized using ECL (enhanced chemiluminescence; GE Healthcare, Australia).

2.4. Immunoisolation of GLAST associated protein

Immunoisolation of GLAST associated proteins was done according to the protocol recommended by Pierce CrossLlink immunoprecipitation kit (Thermo Scientific). The acetone-precipitated protein isolated from human prefrontal cortex was re-suspended in CHAPS and 0.5% sodium deoxycholate buffer. For pre-cleaning of the lysate, 80 µL of the control agarose resin slurry was added to 1 mg of extracted proteins followed by incubation at 4 °C for 1 h and a passage through spin column. The flow through the Pierce spin column, which can be capped and plugged with a bottom plug for incubation or unplugged to remove the supernatant by centrifugation at 1000*g* for 1 min, was used for immunoisolation. The protein A agarose slurry (20 µL) was washed twice with 200 µL PBS buffer, and incubated with 100 µL GLAST

Table 1
Demography of the subjects used in the study.

TRC ID (Case)	Gender	Age (Years)	PMI (h)	pH	Cause of Death
Control					
650^{ns}	M	37	14.5	6.46	Cardiomyopathy
$582^{cs\ (32)}$	M	50	30	6.37	Coronary Artery Disease
$583^{es\ (44)}$	M	59	40	6.53	Ischaemic Heart Disease
$442^{cs\ (23)}$	M	63	24	6.94	Atherosclerotic coronary heart disease
$603^{es\ (25)}$	M	73	38.5	6.28	Acute Myocardial infarction
278^{nk}	M	73	10	6.28	Respiratory arrest
Average		**59 ± 6**	**26 ± 5**	**6.48 ± 0.10**	
Alcoholics					
332^{nk}	M	41	54	6.70	Epilepsy and chronic alcoholism
$192^{cs\ (36)}$	M	54	17	6.41	Cardiomyopathy
$597^{cs\ (62)}$	M	59	35	6.57	Coronary artery thrombosis
$533^{cs\ (150)}$	M	63	25.5	6.21	Ischaemic heart and lung disease
$182^{cs\ (50)}$	M	66	11.5	6.14	Respiratory Arrest
512^{ns}	M	73	43.5	6.59	Coronary artery atheroma
Average		**59 ± 5**	**31 ± 6**	**6.44 ± 0.09**	

Legend: PMI = post-mortem interval (post-mortem delay in hours). The superscript attached to the case numbers indicates the smoking status: cs = current smoker; ns = non-smoker; es = ex-smoker (no smoking for at least a year prior to exitus); nk = not known. The number in parentheses next to the superscript signifies the "pack years" where one pack (20 cigarettes) per day smoked over a period of one year corresponds to one pack year.

antibody prepared in PBS (10 µL GLAST antibody +85 µL H_2O + 5 µL 20X PBS) at 25 °C for 30 min on a mixer. In parallel, 100 µL of rabbit IgG peroxidase secondary antibody with the same concentration of IgG as that of GLAST antibody was similarly prepared as the negative control. The supernatant was discarded and the beads were washed three times with 300 µL PBS, followed by incubation with 50 µL disuccinimidyl suberate (DSS) solution (2.5 µL 20 X PBS + 38.5 µL H_2O + 2.5 mM DSS in DMSO) at 25 °C for 45–60 min on a mixer. After removing the supernatant, the beads were washed three times with 50 µL of 100 mM glycine (pH 2.8), followed by three washes with PBS. About 500 µg of pre-cleaned protein extract was passed through the columns (either anti rabbit-GLAST) or a negative control rabbit -IgG). The columns were washed five times and eluted with acidic buffer (pH = 3). The elution was neutralized with high concentration of Tris-HCl buffer.

2.5. SDS-PAGE and in-gel digestion

The eluted proteins were resuspended with SDS-loading buffer (50 mm Tris-Cl, pH 7.5, 150 mm NaCl), solubilized with gel loading buffer. The mixture was heated at 95 °C for 5 min and duplicate samples were loaded onto 12% SDS-PAGE. One set of samples was stained with Coomassie Brillant Blue (CBB) and the other set was used in WB to confirm the presence of GLAST proteins immunoprecipitated (IP) complexes. The IP positive lanes were cut into 2–4 mm pieces and subjected to proteomic analysis as described previously (Kashem et al., 2007, 2008, 2009a). In brief, gel bands were excised and de-stained using 25 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN) overnight at room temperature. The gel portions were then dehydrated using 100% ACN. Each gel piece was incubated with 12.5 ng/ml trypsin (Roche, sequencing grade) buffer (sodium bicarbonate buffer) for 45 min at 4 °C. After several washings, the gel piece was incubated at 37 °C overnight in 25 mM NH_4HCO_3/0.1% trifluroacetic acid buffer. The peptide mixtures were purified from the supernatant using C_{-18} purification tips (Eppendorf).

2.6. Nano-HPLC MS/MS analysis

Purified peptides were resuspended in 0.1% formic acid (FA) and separated using an Eksigent nanoLC Ultra 2D Plus system coupled to an AB SCIEX (Framingham MA) TripleTOF 6600 mass spectrometer. Peptides were initially loaded onto a 300 µm × 10 mm I.D. ProteCol™ C18 P-120 3 µm HPLC trap column (SGE Analytical Science, Ringwood, Australia) in 2% MeCN, 0.1% FA at 10 µL/min for 10 min. Peptides were eluted by altering the composition of the mobile phase from 97% buffer A (0.1% FA) to 40% buffer B (99.9% MeCN, 0.1% FA) over 95 min at 300 nL/min directly onto an in-house packed 30 cm X 75 µm I.D. ReproSil-Pur 120 C18 AQ-3 µm column (Dr Maisch, Ammerbuch-Entringen, Germany) for separation immediately prior to injection. The TripleTOF 5600 was operated in data independent acquisition mode with the top 15 precursor ions (fulfilling the criteria of charge state between +2 and + 5, minimum intensity of 120 cps, and m/z between 350 and 1250) selected for MS/MS. Selected ions were entered into an exclusion list for 12 s after 1 occurrence. For MS scans, a mass window of 350–1500 m/z and maximum accumulation time of 250 ms was used, while MS/MS scans were conducted using a mass window of 100–1500 m/z, accumulation time of 200 ms, resolution set to unit and with the options for rolling collision energies and adjustment of collision energies.

3. Results

Analysis of the post-mortem human prefrontal cortex by western blotting indicated that GLAST expression was increased 1.6-fold in the extracts of alcoholic brains relative to controls (Fig. 1A and B). To resolve the distinct sub-pools of GLAST within our samples we have extracted protein separately as cytosolic and microsomal fractions (which would include plasma membranes; PM) and studied GLAST expression in both extracts. Alcohol appeared to cause a moderate if any increase (not statistically significant at $P < 0.05$) in GLAST expression in cytosolic fraction but the increase was more pronounced (2.2 fold) in the plasma membrane fraction (Fig. 1A and B). GLAST (EAAT1) is normally expressed in astrocytes (review: Šerý et al., 2015) but our data come from whole tissue, including both astrocytes and neurons (as well as from other structures present in brain such as oligodendrocytes and blood vessels). The data in Fig. 1 are, therefore, best interpreted as virtually all of the alcohol-induced GLAST being located in astrocytic plasma membranes.

The GLAST protein complex was immunoisolated by anti-GLAST antibody and the isolated protein was further analysed through shotgun proteomics. The analysis detected 48 proteins in anti-rabbit GLAST samples and 10 proteins were in rabbit IgG (negative control); these 10

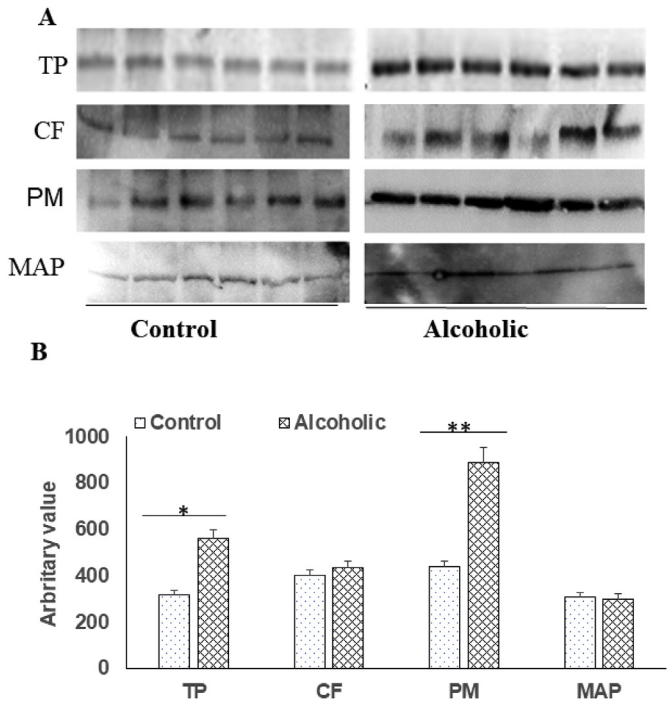

Fig. 1. Immunological analysis of fractions extracted from the prefrontal cortex of control and alcoholic human post-mortem tissues (Fig. 1A. TP, total tissue protein; CF, cytoplasmic fraction; PM, plasma membrane fraction; MAP, microtubule-associated proteins (internal control). Bands were digitized and quantified (Fig. 1B). Columns represent the mean ± SEM (n = 6 per group; significantly different from control at *P < 0.05; **P < 0.01 by paired t-test).

proteins were eliminated as not uniquely related to GLAST. The remaining 38 proteins were considered specifically bound by GLAST (Table 2). These GLAST-associated proteins can be broadly classified as being involved in cell structure (6 proteins; 16%), energy and general metabolism (18 proteins; 47%), neurotransmitter (glutamate and GABA) metabolism (three proteins; 8%), signalling (6 proteins: 16%), neurotransmitter storage/release at synapses (three proteins; 8%) and calcium buffering (two proteins; 5%).

4. Discussion

4.1. GLAST (EAAT1) in alcoholism: altered glutamatergic neurotransmission or perturbed energy metabolism?

Ethanol can alter glutamatergic neurotransmission (reviews: Tsai et al., 1995; Dodd et al., 2000; Spanagel, 2009) and this can include effects on glutamate transporters. Glutamate transporters have a crucial role in removing the excess of glutamate released at glutamatergic synapses (reviews: Danbolt, 2001; Balcar, 2002; Danbolt et al., 2016) and their altered expression would be strongly indicative of major functional changes in glutamatergic synaptic neurotransmission. Indeed, increased GLAST (EAAT1) gene expression accompanies alcohol consumption in rats following prolonged intermittent brain alcohol exposure (Rimondini et al., 2002), and an increased GLAST (EAAT1) immunoreactivity has also been reported in post-mortem brain tissue from alcoholics (Flatscher-Bader and Wilce, 2008). This implies that there could be a close link between GLAST (EAAT1) expression and ethanol consumption. In fact, it has been suggested that a mutation in *Per2* (periodic clock gene 2) which has been associated with excessive drinking in humans may lead to altered GLAST (EAAT1) expression (Spanagel et al., 2005) and such changes could result in an increase in extracellular glutamate and escalation of voluntary alcohol consumption (Spanagel et al., 2005). Thus perturbed GLAST (EAAT1) expression could lead to alcoholism which could, in turn, result in major – probably compensatory – changes in GLAST (EAAT1) abundance and distribution. This is consistent with the present findings, indicating significant increases in GLAST in human alcoholic prefrontal cortex with the observed increases in GLAST (EAAT1) particularly pronounced in plasma membrane fraction. Could higher levels of GLAST (EAAT1) have any additional effects beyond glutamatergic neurotransmission, perhaps impacting proteins participating in activities other than rapid inactivation of synaptically released neurotransmitters? Here, we show that GLAST (EAAT1) interacts with – and could, therefore, potentially influence – 38 proteins of various functional classes (Table 2). The most frequent among them are the proteins involved in energy and neurotransmitter metabolism.

This last observation is particularly intriguing and reinforces other recent findings. As ethanol abuse in humans is generally thought to be associated with the loss of brain cells (review: de la Monte and Krill, 2014), one would expect that most of the ethanol-affected proteins would be those involved in cell proliferation, apoptosis, necrosis and perhaps defence mechanisms against oxidative stress; yet many of the proteins identified as altered by ethanol exposure are actually involved in energy metabolism (humans: 100 out of 238 i.e. 42%; Kashem et al., 2016 and the references cited thereof; rat neural stem cells: six out of 28, i.e. more than 20%; Kashem et al., 2018). Thus the adverse effects of ethanol on human brain tissue may be, to a significant extent, mediated by ethanol-induced disturbances of energy production and utilization. Increased levels of GLAST may, therefore, lead to additional interference with reactions essential for normal energy production and utilization. This would be particularly so, if the energy-related enzymes, normally present in cytosol or associated with mitochondria, were sequestered (and, therefore, potentially inactivated) by the membrane-bound GLAST, which is particularly increased in the alcoholic tissue (Fig. 1). It should also be noted that ethanol affects energy metabolism even in the short term, when no effects on GLAST expression would be expected (Rae et al., 2014).

4.2. GLAST interactome: showing what we know and what we don't. Limitations

Na^+/K^+-ATPase that has been shown to interact functionally with glutamate transport both in cultured astrocytes and in brain cortex tissue in vitro (Pellerin and Magistretti, 1986; Nanitsos et al., 2004; Nguyen et al., 2010) was among the proteins interacting with GLAST. There is much evidence that GLAST interacts with cytoskeletal proteins and this interaction may or may not involve phosphorylation by protein kinase C (Susarla et al., 2004, 2003; Nguyen et al., 2009; Sheean et al., 2013) which also appears among the GLAST-interacting proteins. The presence of GFAP among the structural proteins interacting with GLAST (EAAT1) provides additional supporting evidence for its role in GLAST (EAAT1) anchoring by cytoskeleton in astrocytes and its significance for brain susceptibility to damage as reported earlier (Sullivan et al., 2007). Thus, the overall pattern of GLAST-protein interactions is consistent with what is known of GLAST locations, movements and functions in the cell but points to an intriguing possibility that GLAST (EAAT1) can influence energy metabolism (cf. also Robinson and Jackson, 2016). Interestingly, several proteins known to be involved in the vesicular release of neurotransmitter turned out to be interacting with GLAST. This is not easy to explain in functional terms, since most of the studies suggest that GLAST (unlike GLT1) is located exclusively in glial cells and not in neurons (reviews: Danbolt, 2001; Šerý et al., 2015; Danbolt et al., 2016). Obviously, one must be careful when interpreting the data obtained by the present technique ("interactomics"). Not all interactions detected in homogenized tissue must necessarily occur and be functional in vivo (Robinson and Jackson, 2016).

Table 2
Proteins identified by shot gun proteomics of GLAST immuno-isolated protein complex. GLAST-protein complex was digested with trypsin and identified by LC-MS/MS.

Protein Name	Accession #	Score	Mol. Mass	Pept. Matched	Seq. Cover	Function (if relevant)
Tubulin (b) 2A	gi/4507729	1301	49.87	24	15	Mitosis, Cell division, Cytoskeleton, intracellular transportation
Tubulin (b) 4A	gi/2161322	1208	49.55	22	14	
Tubulin (b)-5	gi/7106439	1003	49.63	20	15	
Tubulin (a) 1A	gi/6755901	834	50.1	13	12	
Glial fibrillary acidic protein	gi/16265836	911	49.78	29	16	CNS development, cell communication
Vimentin	gi/340234	64	35.1	2	2	Cell structure
ATP synthase (b)	gi/32189394	1091	56.5	20	16	Transmembrane transport of various molecules, such as ions and phospholipids, using ATP as a source of energy
ATP synthase (a)	gi/4757810	1014	59.71	20	17	
Na^+/K^+-ATPase (b)	gi|806754	130	34.8	2	2	Na^+,and K^+-exchange pump, provides the ion gradients driving glutamate transport
Malate dehydrogenase	gi|5174539	370	36.4	8	8	Reversibly catalyzes the oxidation of malate to oxaloacetate
Aldolase	gi|312137	341	39.4	6	8	Energy metabolism
L-Lactate dehydrogenase B	gi4557032	317	36.62	7	8	Energy metabolism
Isocitrate dehydrohenase	gi|5031777	182	39.56	8	8	Energy metabolism
Aldehyde dehydrogenase	gi|178390	86	56.34	3	3	Energy metabolism
Enolase 1	gi/203282367	485	47.08	8	8	Energy metabolism
Neuron specific Enolase	gi/55669903	468	47.93	7	7	Energy metabolism
Glyceraldehyde-3-phosphate dehydrogenase	gi|31645	3036	36.03	58	13	Energy metabolism
Pyruvate dehydrogenase	gi|189754	264	39.22	8	5	Energy metabolism
Transaldolase	gi|5803187	135	37.5	6	5	Energy metabolism
Glutamine synthetase	gi|31833	41	42.1	2	2	Glutamate-glutamine cycle
Glutamate dehydrogenase	Gil4885281	221	61.36	5	5	Glutamate metabolism
4-aminobutyrate aminotransferase	gil602706	134	56.52	3	3	GABA metabolism
Phosphoserine aminotransferase	gi|10863955	121	35.1	3	3	Metabolism
Ketimine reductase (mu)	gi|4503065	512	33.7	11	8	Metabolism
Dehydropyrimi-dinase	gi/62087970	518	68.14	6	6	Metabolism
NADH dehydro-genase	gi|4758768	193	40.7	3	3	Energy metabolism
Sirtuin-2	gi|13775600	175	53.9	4	4	Signalling
Quinone oxidoreductase	gi|13236495	137	35.1	3	3	Metabolism
Calcineurin	gi|24158960	83	42.8	2	2	Calcium buffering
Calcium/calmodulin dependent protein	gi/4836793	159	60	5	4	Calcium buffering
Guanine nucleotide-binding protein G(o)	gi|10567816	553	51.1	12	9	Signalling
Protein kinase C	gi|2430822	45	50.9	3	3	Signalling
Serine/threonine-protein phosphatase 2B	gi|6715568	83	58.65	2	2	Signalling
Rab GDI alpha	gi|624873	116	50.39	4	4	Signalling
Protein phosphatase 3	gi|119626528	83	47.08	2	2	Signalling
Synaptophysin	gi|899301	89	32	2	2	Neurotransmitter storage/release
Synaptotagmin-1	gi|5032139	243	47.5	7	6	Neurotransmitter storage/release
Syntaxin-1B	gi|6981600	104	33.2	3	3	Neurotransmitter storage/release

5. Conclusion

Overall, our findings suggest that the complexity of the GLAST interactome that we identify in this study may mean that any alcohol-related changes in GLAST expression and cytoplasmic versus plasmalemmal distribution that we have found are likely to have widely ramified effects on astrocyte biology and function. It is unlikely that the changes in glutamate transport will be the only parameter that is impacted since the co-associated proteins are also likely to change their cellular distributions and abundance if the molecular interactions with GLAST that we identify are indicative of functional biologically relevant interactions at the level of molecular pathways. Accordingly, the data presented here may serve to expose further molecular pathways – such as energy metabolism – that may be significant when studying the effects of alcohol on brain. Conversely, some of the interactions may be indicative of a convergence in mechanisms with other types of brain injury where profound changes in proteins such as glutamine synthetase (an enzyme in glial cells to which GLAST supplies glutamate; Table 2) that have also been described both in alcoholism and neurodegenerative disease (Matsuda-Matsumoto et al., 2007; Lee et al., 2010), observations which may highlight pathological changes in astrocytes in multiple disease states.

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