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Synaptic proteome changes in the superior frontal gyrus and occipital cortex of the alcoholic brain

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Abstract

Cognitive deficits and behavioral changes that result from chronic alcohol abuse are a consequence of neuropathological changes which alter signal transmission through the neural network. To focus on the changes that occur at the point of connection between the neural network cells, synaptosomal preparations from post-mortem human brain of six chronic alcoholics and six non-alcoholic controls were compared using 2D-DIGE. Functionally affected and spared regions (superior frontal gyrus, SFG, and occipital cortex, OC, respectively) were analyzed from both groups to further investigate the specific pathological response that alcoholism has on the brain. Forty-nine proteins were differentially regulated between the SFG of alcoholics and the SFG of controls and 94 proteins were regulated in the OC with an overlap of 23 proteins. Additionally, the SFG was compared to the OC within each group (alcoholics or controls) to identify region specific differences. A selection were identified by MALDI-TOF mass spectrometry revealing proteins involved in vesicle transport, metabolism, folding and trafficking, and signal transduction, all of which have the potential to influence synaptic activity. A number of proteins identified in this study have been previously related to alcoholism; however, the focus on synaptic proteins has also uncovered novel alcoholism-affected proteins. Further exploration of these proteins will illuminate the mechanisms altering synaptic plasticity, and thus neuronal signaling and response, in the alcoholic brain.

Keywords

human brain; occipital cortex; superior frontal gyrus; synapse

1 Introduction

Misuse of psychoactive substances is placing an increasing burden on healthcare and societal welfare systems worldwide and although public attention is drawn to the ever-

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increasing use of illicit drugs, the greatest impact on our society is still made by a licit drug: alcohol. A World Health Organization study [1] found that, world-wide, 3.2% of deaths are alcohol-related, and there are distinct commonalities between countries in terms of consumption patterns and their resulting cognitive and neurological defects. Repeated and long-term alcohol abuse leads to cerebral and cerebellar damage causing adaptive changes manifesting as tolerance and dependence [2], and also general cognitive decline in functions such as learning capacity, memory, decision making and perceptual motor skills [3]. Many of these executive functions are controlled by the frontal cortex; hence, this area is considered severely affected by alcoholism. In addition, this area is one of the specific regions susceptible to alcoholism-induced neuron loss [4,5], showing 23% loss in alcoholics when compared to controls [6]. Other areas, such as the occipital cortex (OC), do not lose grey matter in response to alcoholism [7] and do not exhibit obvious functional changes such as altered visual interpretation or blindness, so this brain region is considered ‘spared’ from the affects of alcoholism. However, this term is specific to cognitive/functional effects as reduction of dendritic arborisation is extensive throughout the brain, including within the OC, with subsequent loss of synaptic connections, although this does not cause volume loss [8]. fMRI has revealed that alcoholics show a decreased OC response to visual stimulation, a response not observed in the frontal cortex despite significant activation of this area [9]. Additionally, acute alcohol administration decreases glucose metabolism within the brain, with the strongest effects in the OC [10,11].

Microarray [12,13] and proteomic [14,15] studies have clearly demonstrated substantial transcriptional and translational changes in the frontal cortex of the alcoholic brain, although the regulatory mechanisms behind the pathological changes seen in this area remain unknown. Apart from the current study, no proteomic or transcriptomic analysis has been done of the OC in relation to alcoholism.

All neurological changes that occur as a result of chronic alcohol abuse alters the transmission of signals through the neural network, which, in turn, disturbs or modifies the brain's response. The synapses are the connection between the cells of the neural network; synaptic plasticity determines the properties and intensity of the signal and response, so modification of synaptic components are likely to induce significant changes to neuronal function. In an attempt to tease out the mechanism underlying synaptic dysfunction in alcoholism, we used a fractionation approach to isolate synaptosomes from susceptible (SFG) and spared (OC) brain regions from alcoholics and controls. This approach has shown that there is significant alteration of metabolic and energy production pathways in both the SFG and OC of alcoholics, with some novel vesicle transport and signal transduction pathways also modified. Unexpectedly, the OC exhibited almost twice as many protein changes than the SFG; considering the lack of functional changes that occur in this area, this response may be due to a better protective response in the OC or a lack of protection in the SFG. This result and the specific protein changes are discussed in relation to altered synaptic activity in the alcoholic brain.

2 Materials and methods

2.1 Case Selection

Subjects were categorized according to alcohol intake. Alcoholics were defined by National Health and Medical Research Council/WHO criteria as individuals who had consumed an average of more than 80 g ethanol/day throughout most of their adult life; the alcohol consumption of controls was < 20 g ethanol/day. Cases with multiple drug use or Wernicke-Korsakoff syndrome were excluded. Control cases had no history of brain dysfunction and no significant brain abnormalities were present upon post-mortem examination. Six cases from control and alcoholic groups were chosen and matched as closely as possible for age,

sex and post-mortem delay (PMD). The clinical details of the chosen cases are described Table 1.

Samples were collected by qualified pathologists from the Brisbane node of the National Health and Medical Research Council (NHMRC) Brain Bank and the Tissue Resource Centre at the University of Sydney, Australia. Full ethical clearance and informed written consent was obtained from the next of kin.

2.2 Protein extraction

Synaptosomes were prepared essentially as per Dodd et. al. [16] with some changes. Brain tissue (approximately 0.5 g) was thawed in 0.32 M sucrose at 37°C, immediately transferred to 10 volumes of ice-cold 0.32 M sucrose, and homogenized at 500 rpm with in a motor driven Teflon-glass homogenizer at 4°C. Sample was centrifuged at 755 g at 4°C for 10 min to remove debris, then the supernatant was centrifuged at 19000 g at 4°C for 20 min. The pellet was resuspended in 10 volumes of 0.32 M sucrose then layered over a sucrose gradient (consisting of a 1.2 M sucrose layer under a 0.8 M sucrose layer) and centrifuged at 75000 g at 4°C for 1 h. The synaptosomal fraction was carefully removed from the interface between the 0.8 M and 1.2 M sucrose layers and frozen at −80°C for storage until use.

Aliquots of the synaptosomal fraction were purified using the 2D-Cleanup kit (GE Lifesciences, Princeton, NJ, USA) then resuspended in CyDye (GE Lifesciences) labeling buffer containing 30 mM Tris, 7 M urea, 2 M thiourea and 4% (w/v) CHAPS. Each sample was quantified using the 2D-Quant kit (GE Lifesciences).

2.3 2D-Differential In-gel Electrophoresis (DIGE)

2.3.1 CyDye labeling—16 μg aliquots of each protein were labeled with CyDye fluors as described in the CyDye DIGE Fluors (minimal dye) Labeling Kit (GE Lifesciences), with some variations. 16 μg of each sample was made up to 9 μL with CyDye labeling buffer (above); 350 μM CyDye working solution was prepared with fresh DMF, then 175 pmol CyDye was added to each sample. After 30 min on ice in the dark, 1 μL 10 mM Lysine was added and the mixture left on ice in the dark for 10 min to stop the reaction. Samples were stored at −80°C until use.

A dye-swapping approach was utilized to minimize the effects of variable CyDye labeling efficiency. In essence, half of each group (control or alcoholics) from each brain region (superior frontal gyrus, SFG or occipital cortex, OC) was labeled with Cy3, and the other half was labeled with Cy5. An internal standard (IS) was created by mixing 20 μg each sample from both brain regions and both groups (alcoholics and controls) then divided into 12 × 16 μg aliquots and labeled with Cy2.

2.3.2 2-Dimensional Electrophoresis (2DE)—One Cy3 labeled sample and Cy5 labeled sample of the same brain region but different group (control or alcoholic) were mixed with one IS Cy2 labeled aliquot. 10.5 μL sample buffer (8 M urea, 4% (w/v) CHAPS, 130 mM DTT, 2% (v/v) IPG buffer 3-11 non-linear (NL)) was added, then the sample was brought to 408 μL with rehydration buffer (8 M urea, 4% (w/v) CHAPS, 13 mM DTT, 1% (v/v) IPG buffer 3-11 NL). The final solution contained 15.7 mM DTT and 1% (v/v) IPG buffer 3-11 NL. After thorough mixing the samples were separated by isoelectric focusing (IEF) on a 24 cm pH 3-11 NL Immobiline DryStrip immobilized pH gradient (IPG) (GE Lifesciences), using the Ettan IPGphor II IEF system (GE Lifesciences) with a 12 h 30 V active rehydration step followed by 2 h of prefocusing (1 h each at 500 V then 1000 V), then a final focusing step at 8000 V until 40000 total Vh was reached. IPG strips were then incubated in equilibration buffer (50 mM Tris-Cl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2%

(w/v) SDS, 0.002% (w/v) bromophenol blue) containing 0.1% (w/v) DTT for 15 min, then 0.25% (w/v) iodoacetamide for 15 min. Equilibrated IPG strips were fixed into the top of 24 cm 10% acrylamide SDS-PAGE gels using 0.5% (w/v) agarose in running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) with 0.002% (w/v) bromophenol blue. Separation was performed on an Ettan DALT 12 separation unit (GE Lifesciences) running at 2 W/gel for 30 min, followed by 5 W/gel for 30 min, then 10 W/gel for 5 h.

2.4 Comparative analysis

Gels were scanned on a Typhoon 9400 scanner (GE Lifesciences) at three wavelengths to record the fluorescence of each of the three Cy Dyes in the gel. Images were cropped and rotated in Image Quant (BioRad, Hercules, CA, USA), then imported into the TT900 S2S (NonLinear Dynamics, Newcastle upon Tyne, UK) image alignment module for gel/spot alignment of all gels to each other. Aligned gels were imported into Progenesis PG240 (NonLinear Dynamics) then automatically analyzed using the SameSpots module to accurately compare the same protein spot in each gel. Automatic analysis included background subtraction and radiometric normalization. This method of normalization is standard for DIGE experiments; it expresses each spot's volume as a ratio to its matching spot on the IS image, multiplied by a ‘normalizing factor’ (the ratio of the total spot volume of the IS to the total spot volume of the analysis (i.e. Cy3 or Cy5) gel). Although identical protein amounts were loaded onto each gel, the IS acts as an additional control since it is identical on all gels; hence, the normalizing factor controls for any unequal protein loading that may occur. The Progenesis software automatically generates an ‘average’ gel for each group (alcoholics or controls) and region (SFG or OC) combination. In essence, the normalized volume of each spot from all six gels in one group/region combination is averaged to create a virtual gel comprising mean ± error value for each spot. This ‘average’ gel is then used for comparison to the other group/region combinations within the Progenesis software to determine expression differences.

o determine statistical significance the normalized volumes of each spot from all gels were analyzed in Statistica (Statsoft, Tulsa, OK, USA) using a repeated measures ANOVA with post-mortem delay and age as covariants. To correct for multiple testing, the resulting ANCOVA values were subjected to a Newman-Keuls post-hoc test; the resulting *P* values were used as the measure of statistical significance. Only proteins with an expression difference greater than 1.2 and a Newman-Keuls P value ≤ 0.05 were analyzed in this study.

2.5 Trypsin digest

For protein excision, an additional 2DE gel was run with 400 μg protein as described above excepting the CyDye labeling. For these preparative gels, samples from a single group/region combination were mixed to obtain enough protein. After the second dimension, gels were fixed in 50% (v/v) ethanol/2% (v/v) o-phosphoric acid overnight, washed three times in water, then stained in 17% (w/v) ammonium sulfate, 3% (v/v) o-phosphoric acid, 34% (v/v) methanol and 0.066% (w/v) colloidal Coomassie Brilliant Blue G250 (Fluka, Buchs, Switzerland) for two days. Gels were scanned using a standard flat-bed scanner and spots previously determined as exhibiting a statistically significant expression difference were visually identified by comparison with gel images used in the analysis. These protein spots were excised from the preparative gel and stored in 1% acetic acid [17] until use.

Acrylamide plugs were washed and dehydrated by 15 min washes in 25 mM ammonium bicarbonate/5% (w/v) acetonitrile (twice) and 25 mM ammonium bicarbonate/50% (w/v) acetonitrile (HPLC grade; Sigma, St Louis, MO, USA). Plugs were dried at room temperature under vacuum with centrifugation (SPD SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min, then rehydrated in 10 μL 20 ng μL-1 acidified trypsin

(Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 4°C for at least 60 min. Excess trypsin solution was removed and replaced with 5 μL 25 mM ammonium bicarbonate, then the samples were left at 37°C for at least 16 hours for digestion to occur. Peptides were extracted first with 15 μL 0.1% (v/v) trifluoroacetic acid (TFA), then twice more with 30 μL 5% TFA in 50% (v/v) acetonitrile. Extracted peptides were dried at room temperature under vacuum with centrifugation (SPD SpeedVac; Thermo Fisher Scientific, Waltham, MA, USA) for 3 hours, and then stored at 4°C.

2.6 MALDI-TOF mass spectrometry

Dried peptides were resuspended in 1.5 μL matrix solution (5 μg/μL α-cyano-4-hydroxy cinnamic acid (CHCA), 60% (v/v) acetonitrile, 0.1% (v/v) TFA) then spotted directly onto the MALDI-TOF target (stainless steel sample plate; Applied Biosystems, Foster City, CA, USA) with 0.5 μL calibration standard mixture (5 fmol/μL angiotensin I (Sigma), 5 fmol/μL ACTH(1-17) (Sigma), 3.75 fmol/μL ACTH(18-39) (Sigma), 60% (v/v) acetonitrile, 0.1% (v/v) TFA). Spots were left to dry for 30 min. Peptide mass fingerprints (PMFs) of masses between 800 and 5000 Da were obtained for each peptide mixture using a Voyager DE-STR BioSpectrometry Workstation (Applied Biosystems) in positive reflector mode with delayed extraction and a mass gate of 500 Da; the accelerating voltage was set at 25 000 V with a grid voltage of 68% and a delay time of 300 nsec. Laser intensity was optimized for each peptide mix. The resultant spectra were processed using Data Explorer software (Applied Biosystems). Each PMF was baseline corrected using the default parameters of the Advanced Baseline Correction tool, and peaks were deisotoped to create a monoisotopic spectrum using default parameters of the deisotoping tool. Each spectrum was calibrated internally using at least two of the calibration standards (Angiotensin I, 1296.6853 Da; ACTH(1-17), 2093.0867 Da; ACTH(18-39), 2465.1989 Da).

2.7 Database searching

20 to 40 of the most abundant masses in the resultant PMF were used to search the NCBInr database on the web-based version of MASCOT (http://www.matrixscience.com) to determine its identity by comparison of the experimentally determined peptide masses with theoretical masses calculated for all proteins in each database. Search stringency was set at a high level by limiting the difference between a theoretical peptide's mass and that of the experimental peptide to only 30 ppm (0.003% difference). During the search, carbaminomethyl modification was assumed for all peptides, and oxidation of methionine was optional. A positive protein match was accepted with a significant probability score (>65 for the Homo sapiens NCBI database, $p<0.05$), a sequence coverage >10% for large proteins (>80 kDa) or >15% for smaller proteins (<80 kDa) and more than 5 unique peptide matches. Other factors were also considered, such as comparison of the experimental and theoretical molecular weights and isoelectric points; however, these were not used as absolutely defining criteria, as certain proteins could be under the influence of factors that may alter those characteristics, such as post-translational modifications, degradation, or processing.

3 Results

3.1 Comparison of alcoholics to controls

To explore the changes induced by alcoholism at the synapse, we analyzed protein extracts from post-mortem human brain tissue of six alcoholics and six matched controls by two dimensional differential in-gel electrophoresis (2D-DIGE). Protein expression profiles of synaptosomal extracts were generated for two brain regions from each subject: the superior frontal gyrus (SFG), a region severely affected by alcoholism, both functionally and

pathologically, and the occipital cortex (OC), which shows little functional change in alcoholics.

Six gels were generated for each brain region, each gel containing a non-alcoholic control sample, an alcoholic sample and an internal standard; all gels from both regions were analyzed together. Approximately 1700 distinct protein spots were present on all gels and used in the analysis. To control for gel-to-gel variation, the volumes of each spot were normalized using standard DIGE Radiometric normalization (see section 2.4 in Materials and Methods). The normalized volumes from all spots in each group (Alcoholic-SFG, Alcoholic-OC, Control-SFG, Control-OC) were then analyzed with a repeated measures ANOVA taking into account the 'within effects' that occur due to the fact that two areas (the repeated measure) were sampled from each case. Additionally, the effect of varying post-mortem delays and age were minimized through the use of these values as covariants during the ANOVA. To counteract the effects of multiple testing, all ANCOVA values were subjected to the Newman-Keuls post-hoc test to generate the *P* values used to define statistical significance ($P \leq 0.05$).

3.2 Comparison of alcoholics to controls in the SFG or OC

To identify protein expression changes between alcoholics and controls, each brain region was analyzed in isolation (i.e. alcoholic SFG was compared to control SFG; alcoholic OC was compared to control OC). 49 proteins showed a statistically significant difference > 1.2 fold (*P* value ≤ 0.05, Student's t-test) in the SFG; 94 such proteins were identified in the OC (Table 2). Slightly more than half of differences are between 20 and 50% (1.2 to 1.5 fold; Table 2a), and, even with the small number of subjects used, these differences are highly significant and so were considered to be a realistic reflection of differences in the alcoholic brain. Considering the highly regulated state of the brain, small changes in brain-protein levels are likely to result in a large affect on brain function. Twenty-three proteins were significantly different between alcoholics and controls in both brain regions.

3.3 Comparison of SFG to OC in alcoholics or controls

The data was further analyzed to identify expression changes specific to each brain region. In this analysis, the SFG was compared to the OC in either alcoholics or controls (i.e. alcoholic SFG was compared to alcoholic OC; control SFG was compared to control OC). Approximately two-thirds of the proteins in this comparison exhibited small differences (between 20 and 50%; Table 2b), and there were one-third more changes between the brain regions in controls (138 changes) compared to alcoholics (92 changes).

3.4 Identification of differentially regulated proteins

Fifty six of these proteins were identified by MALDI-TOF mass spectrometry (Table 3). Several proteins identified in multiple spots were also regulated (Table 3), which is not an uncommon observance on 2D gels of brain proteins [18-20]. The technical artifact carbamylation is often held culpable for presence of multiple spots on a 2D gel, however the process of this modification requires a breakdown product of urea that is only created at temperatures over 37°C – a situation that is rare during proteomic procedures [21,22]. Rather than artifacts, these isoforms represent potential post-transcriptional (e.g. splice variants) or post-translational (e.g. phosphorylation) modifications.

Dynamin-1 was identified in a line of multiple spots on the 2DE gel, some of which were differentially regulated between alcoholics and controls. These proteins exhibited a range of isoelectric points (pI) between approximately 6.3 and 7.3 at a molecular weight of 96 kDa (Figure 1A). When comparing alcoholic SFG to control SFG, five of these isoforms were reduced in alcoholics with a Newman-Keuls *P* value <0.05 (protein #61, 37, 42, 50 and 40;

Figure 1B) and four others had a Newman-Keuls *P* value <0.09 (protein #39, 58, 55 and 46; Figure 1B). Protein #37 was also lower in the alcoholic OC compared to control OC (Newman-Keuls *P* value <0.05; Figure 1B) and proteins 61 and 39 showed a decreasing trend in the OC with Newman-Keuls *P* value <0.09 (Figure 1B).

Dynamin-1 isoforms also show region-specific differences in normal, non-alcoholic brain. Two isoforms (#50 and 40) showed higher expression in the SFG than the OC in control samples (Figure 1B). This region specificity was lost in alcoholics indicating an alcoholism specific change of these isoforms in the SFG. One dynamin-1 isoform showed decreased expression in the SFG compared to the OC in both alcoholics and controls (#39; Figure 1B), indicating that this isoform is not altered in response to alcoholism.

There are five known human dynamin-1 splice variants, three of which have a molecular weight (MW) close to 96 kDa but with slightly different pI values (ENSP00000377219: pI 6.32, 95835.25 Da; ENSP00000345680: pI 6.57, 95980.34 Da; ENSP00000362014: pI 6.73, 97346.97 Da; Ensemble gene report ENSG00000106976). Three isoforms identified on the 2DE gel may represent these splice variants, although this has yet to be determined. The other isoforms may be novel variants or could represent different post-translationally modified forms of dynamin-1.

Several heat shock proteins of the 70 kDa sub-family (HSP70) also showed evidence of multiple isoforms (HSP70-1, protein #62 and 131; HSP70-8, #147, 36, 163, 31); however, unlike the decrease of all dynamin-1 isoforms in alcoholics, some HSP70 isoforms increased, while others decreased (Table 3a). The HSP70s showed the most change between alcoholics and controls in the OC, with only a few isoforms altered in the SFG.

Various HSP70 proteins also showed region specific expression patterns in controls (HSP70-1, #62; HSP70-5, #260; HSP70-8, #147, 36, 163; Table 3b) or alcoholics (HSP70-1, #131; Table 3b). Two HSP70 proteins showed distinct region specific differences in both alcoholics and controls: HSP70-12A, protein #104 and 89, and HSP70-2, #161 (Table 3b).

The two isoforms of DR*P* 2 observed on the 2D gel may represent a truncated and full-length version of the protein, as has been observed in other proteomic studies [23]. The proportion of these two isoforms alters to favor the truncate during prolonged post-mortem storage at room temperature in mouse brain [23], however this isoform swap was not observed in our alcoholic sample; in fact, we observed that both isoforms were higher in the alcoholic OC than in the alcoholic SFG, and the truncate alone was higher in alcoholic SFG without a concomitant decrease of the full length version. In our study it appears that the changes observed in DR*P* 2 are not related to post-mortem storage conditions, but may represent a functionally relevant change in DR*P* 2.

Our data on multiple isoforms of a particular protein are difficult to compare to other 2DE proteomics studies since verification of the isoform under discussion can be problematic. For example, two oppositely-regulated isoforms of HSP70-8 were identified in a total protein extract of the SFG [15]; however, whether these are the same isoforms identified in the current study is unclear. Regardless of proof of identity of the isoform, these studies provide evidence for alteration of multiple protein isoforms by chronic alcoholism.

4 Discussion

Our analysis revealed that alcoholism is causing changes to the synaptic proteome in both the SFG and the OC, with a small subset of proteins altered in both regions. This group of proteins is quite different to those changes that occur in whole tissue proteomes of similar brain regions [14,15,24], a result most likely due to the focus of this study specifically on

synaptosomal proteins. Additionally, we have identified that the visual association area represented by the OC region of the brain is reacting to alcoholism in a very significant way, with our finding that almost twice as many proteins are altered between alcoholics and controls in this region than in the SFG.

This result suggests that the OC is not as spared as previously thought. Considering that the OC receives input from areas of higher brain function such as the frontal cortex and thalamus, these changes could be, in part, a response to the alterations taking place in other brain regions. The brain-wide neuropathological effects of alcoholism may also be causing direct protein changes, although this is not necessarily to the detriment of the OC. The greater response of the OC could be an indication that those changes are induced to increase protection from the effects of alcoholism in this region, resulting in no changes to the functionality of visual processes.

As expected, many proteins altered by alcoholism are involved in energy metabolism or glycolytic pathways. Alcoholism affects various metabolic pathways throughout the body, including the brain, and alcoholism-induced alterations could lead to disruption of essential energy-producing processes, resulting in reduced cellular functionality and possibly cell death. Some altered proteins are involved in aerobic glycolysis and mitochondrial oxidative phosphorylation which supply the brain's high energy requirements through ATP production [25]. Without appropriate control of ATP production by glycolytic and other mitochondrial pathways in the brain, certain highly-used, and thus energy-hungry, areas are bound to be adversely affected – possibly to the point of death due to energy starvation.

The decrease of creatine kinase B (CKB) in the alcoholic SFG (this study), OC (this study), hippocampus [26,27], and cerebellar vermis [26,27] shows that energy metabolism is altered in many parts of the alcoholic brain. The corpus callosum is notable in its differences in that this protein is not altered in the splenium [28], and is increased in the genu [29], suggesting region specific regulation.

CKB is an essential enzyme in energy-hungry tissues such as the brain where it catalyses the phosphorylation of creatine to phosphocreatine. This is not only an energy storage molecule required for rapid ATP synthesis, but can act as a ‘shuttle’ to move the phosphate from the site of consumption to the site of generation [25]. Those brain regions showing reduced CKB are likely to have a reduced capacity for energy storage and production which would severely compromise synapse function.

Changes to the glycolysis proteins fructose-bisphosphate aldolase A and C are specific to the OC, and are interconnected with changes to the cytoskeleton. Aldolases catalyze the cleavage of fructose 1,6-bisphosphate (FBP) to glyceraldehyde 3-phosphate and dihydroxyacetone in glycolysis, but they also interact with several other proteins, including filamentous actin [30], possibly for co-localization of glycolytic proteins [31] such as triosephosphate isomerase [32], another protein altered by alcoholism (this study). In addition to direct interaction with actin, aldolase sequesters the actin filament nucleation protein Wiskott-Aldrich syndrome protein (WASP), which inhibits both WASP and aldolase activity until enough substrate (FBP) is present to dislodge it [33,34]. Actin itself is also decreased specifically in the OC of alcoholics (this study), suggesting that alcoholism may affect the actin dynamics of OC cells directly, through γ-actin, and indirectly, through disruption of aldolase's metabolic function and through WASP.

Altered regulation of cytoskeletal components and associated signaling pathways can cause significant changes to synaptic and axonal function. Dihydropyrimidinase-related protein (DR*P* 2), involved in neuronal repair in the adult brain through control of axonal outgrowth of regenerated neurons, is down-regulated in Alzheimer's disease brain [35], but has

increased oxidation [36,37]. In this disease, loss of non-oxidized DR*P* 2 may increase neurodegeneration by preventing neuronal repair. The increase in DR*P* 2 levels in the OC found in this study may be a compensatory mechanism in response to alcoholism-induced damage as a means to increase axonal growth, repair and regeneration.

Several proteins involved in synaptic transmission were altered by alcoholism. The brain-specific protein, dynamin-1, controls synaptic-vesicle recycling via endocytosis where it is involved in scission of clathrin-coated vesicles from a parent membrane in the pre-synaptic cell [38,39]. It is essential only during the application of a strong or sustained stimulus when exocytosis of neurotransmitter-containing vesicles is extreme and thus requires rapid retrieval of clathrin-coated vesicles via endocytosis to maintain the pool of synaptic vesicles [39]. There were 12 dynamin-1 isoforms identified in both brain regions, but more were significantly lowered in response to alcoholism in the SFG (5 isoforms) than in the OC (1 isoform). Three of these isoforms show region specific expression in non-alcoholic controls indicating that the different isoforms may be responsible for different functions in each region of normal brain. For two isoforms, these expression differences are lost in alcoholics due to significant decrease of these isoforms in the alcoholic SFG region. Alcoholics exhibit defects in cognitive processes controlled by the SFG such as learning and decision-making, thus reduced or impaired synaptic-vesicle recycling, and thus neuronal signaling, due to loss of dynamin-1 in this area may underpin alcoholism's neurodegenerative effects and its general disruption of cognitive function.

The modification responsible for multiple expression forms of dynamin-1 is not known, however each is likely to have a distinct function since those altered by alcoholism do so independent of the modification. In this manner, chronic alcohol misuse may be disrupting multiple synaptic functions through a single protein.

Other studies of the alcoholic SFG either did not report a change in dynamin-1 [15], or found an increase [14]. No other study has shown that multiple isoforms of dynamin-1 are regulated in alcoholics. This disparity is likely due to differences in protein preparation: this study focused on an analysis of enriched synaptic proteins, while the other studies utilized total protein extracts [14,15,24]. By fractionating the synaptosomal proteins we have enriched those proteins which are normally of low abundance within the total protein extract, such as the different isoforms of dynamin-1, and have thus been able to identify differences in the levels of these low-abundant proteins in alcoholics.

Other proteins, such as several HSP70s, showed an alcoholism-induced shift from one isoform to another. Most HSP70 expression is stress-induced [40], and different HSP70s are induced by short-term [41-43] and long-term [14,15,44,45] alcohol consumption; however, some are also expressed at a basal level (e.g., in human brain HSP70-2 [46], HSP70-5 [47], and HSP70-8 [48,49]). The chaperone-related housekeeping roles of constitutively expressed HSP70s work to prevent aberrant protein folding and targeting [40,50]. These functions are subsumed into protective roles during stress, along with the inducible HSP70s, to combat the effects of excitotoxicity (particularly HSP70-1 [51]) and oxidative damage (HSP70-1 [41,42]; HSP70-8 [52,53]). Excessive consumption of alcohol causes both of these stresses: ethanol metabolism pathways produce free radicals that cause oxidative damage in the liver [54,55] and brain [56], and chronic alcohol abuse enhances excitotoxicity through altered regulation of the neurotransmitters γ-aminobutyric acid (GABA) and glutamate, and their receptors [57-59].

Changes to the levels of different HSP70 isoforms may be elicited as a protective response against these stresses. Differently modified isoforms are likely to have slightly varying functions, thus this shift in the predominance of particular isoforms suggests an altered

requirement for specific functional isoforms. When compared to the same region in controls, the alcoholic SFG showed fewer changes to the HSP70 proteins than the OC, potentially leaving it more susceptible to oxidative damage or excitotoxicity. Additionally, the basal level of some HSP70 isoforms in non-alcoholic brain differs between the SFG and OC (Table 3b). Many of these differences are lost in alcoholics further suggesting region-specific changes resulting in loss of HSP70 functionality.

These results give an interesting glimpse into the synaptic-specific changes induced by alcoholism and indicate pathways involved in alcoholism's effect. The differences between the changes that occur in the two brain regions demonstrate that the synapses of the SFG and OC have both been affected by alcoholism to different degrees. Changes to vesicle transport and cytoskeleton proteins are indicative of alcoholism-induced changes to synaptic transmission pathways and could potentially explain alcoholism's neurodegenerative effects and disruptions to cognitive function. Evidence of enhanced protective functions is present in the changes to chaperone levels and isoforms, particularly in the functionally operational OC. Further study of these proteins will further our knowledge of alcoholism's effect on the brain, and also help us to gain insight into mechanisms of neurodegeneration and synaptic loss.

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Abbreviations

SFG	superior frontal gyrus
OC	occipital cortex

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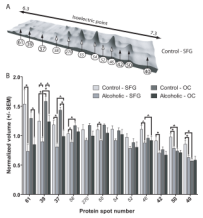

Figure 1.

Dynamin-1. (**A**). A topographic display of the area surrounding the dynamin-1 isoforms on a representative control sample from the SFG. Heights of the projections on the topographic display are representative of intensity of protein amounts. All labeled proteins were identified as dynamin-1; italicized numbers represent those proteins which were not significantly different from the same protein in any other group (significance was determined as those having a *P* value ≤ 0.05, ANCOVA corrected for PMD and age with Newman-Keuls post-hoc test) . (**B**). Graph of average normalized volumes of each protein (derived from the average of 6 biological replicate samples per group), ± SEM. *, comparison of the two indicated groups results in a *P* value ≤ 0.05 (ANCOVA corrected for PMD and age with Newman-Keuls post-hoc test). ^, comparison of the two indicated groups results in a *P* value <0.1 (ANCOVA corrected for PMD and age with Newman-Keuls post-hoc test).

Table 1

Case information.

Sex	Age (yrs)	PMD[a] (hours)	Brain Weight (g)	Cause of Death
Controls				
M	71	4.75	1305	Acute renal failure
M	75	36.5	1333	Sepsis
M	67	67	1320	Myocardial infarction
M	52	61.75	1515	Myocardial infarction
M	89	17	1600	Congestive cardiac failure
F	54	15.75	1200	Pulmonary embolism
Alcoholics				
M	49	16	1420	Dilated cardiomyopathy
M	34	31	1440	Aspiration of gastric contents
M	44	22.5	1540	Not determined
F	46	16.5	1340	Myocarditis, sarcoidosis
M	59	15	1490	Hanging
M	69	36	1540	Coronary atheroma

[a] post mortem delay

Table 2

Breakdown of the numbers of changes between alcoholics and controls in two different brain regions. **A.** The average normalized volume of each spot in alcoholics (six individuals) was compared to the average normalized volume of the same spot in controls (six individuals) in two brain areas; only differences greater than 1.2 fold with a *P* value ≤ 0.05 (ANCOVA with Newman-Keuls post-hoc; values are corrected for post-mortem delay and age) are listed. **B.** The average normalized volume of each spot in the SFG (six individuals) was compared to the average normalized volume of the same spot in the OC (six individuals) in each group (alcoholics or controls); only differences greater than 1.2 fold with a *P* value ≤ 0.05 (ANCOVA with Newman-Keuls post-hoc; values are corrected for post-mortem delay and age) are listed. SFG, superior frontal gyrus; OC, occipital cortex.

A

	Expression changes in ALCOHOLICS compared to CONTROLS			
	SFG		**Occipital**	
Difference	+	−	+	−
±1.2-1.3	10	9	11	18
±1.4-1.5	5	5	12	14
±1.6-2.0	4	10	10	16
>2.0	1	5	6	7
Subtotal	*29*	*20*	*39*	*55*
Total	*49*		*94*	

B

	Expression changes in the SFG compared to the OC			
	ALCOHOLICS		**CONTROLS**	
Difference	+	−	+	−
±1.2-1.3	17	15	18	24
±1.4-1.5	8	21	17	26
±1.6-2.0	8	15	20	21
>2.0	3	5	6	6
Subtotal	*36*	*56*	*61*	*77*
Total	*92*		*138*	

Table 3

Summary of proteins identified by MALDI-TOF mass spectrometry that are regulated in the SFG and/or the OC of alcoholics when compared to controls. Selected proteins with expression changes between alcoholics or controls in either region **(A)** or between each region in either alcoholics or controls **(B)** which were greater than 1.2 fold with a *P* value ≤ 0.05 (ANCOVA, corrected for post-mortem delay and age, subjected to the Newman-Keuls post-hoc test) were identified by mass spectrometry. Italicized numbers indicate those not reaching cut-off parameters for either statistical significance and/or expression change. Note that some proteins are represented in both (A) and (B).

A

		MASCOT MALDI-TOF significance scores[a]					Expression changes in ALCOHOLICS compared to CONTROLS[b]			
							SFG		OC	
Protein #	SwissProt Accession	MASCOT score	Expect score	Hits	% Cov	Identification	Difference	*P* values*	Difference	*P* values*
Metabolism - energy										
57	P12277	191	1.20E-14	15	54	Creatine kinase B-type	**−1.2**	**0.0037**	**−1.2**	**0.0002**
53	P12277	165	4.80E-12	14	44	Creatine kinase B-type	**−1.2**	**0.0112**	**−1.3**	**0.0021**
151	P50213	77	4.00E-03	10	31	Isocitrate dehydrogenase [NAD] subunit alpha	*− 1.0*	*0.6629*	**−1.3**	**0.0303**
Metabolism - glycolysis and glycogen										
16	P00558	95	4.60E-05	8	31	Phosphoglycerate kinase 1	**1.4**	**0.0283**	**1.4**	**0.0385**
126	P04075	87	3.40E-04	8	30	Fructose-bisphosphate aldolase A	*1.1*	*0.3809*	**1.4**	**0.0177**
160	P04075	164	6.10E-12	12	59	Fructose-bisphosphate aldolase A	*1.2*	*0.0990*	**1.5**	**0.0070**
28	P07195	136	5.10E-09	14	41	L-lactate dehydrogenase B chain	*1.2*	*0.0812*	**1.2**	**0.0503**
86	P09972	142	9.70E-10	11	46	Fructose-bisphosphate aldolase C	*1.2*	*0.2999*	**1.8**	**0.0023**
144	P11177	86	4.70E-04	11	36	Pyruvate dehydrogenase E1 component subunit beta	*1.0*	*0.6383*	**−1.2**	**0.0038**
110	P18669	140	2.10E-09	13	55	Phosphoglycerate mutase 1	*− 1.1*	*0.6325*	**−1.3**	**0.0546**
24	P60174	171	1.50E-12	12	61	Triosephosphate isomerase	**1.3**	**0.0119**	1.2	0.0865
Metabolism - oxidative phosphorylation										
122	P21281	154	6.10E-11	14	33	V-ATPase subunit B2	*1.1*	*0.4634*	**1.3**	**0.0234**
141	P21281	70	1.90E-02	8	26	V-ATPase subunit B2	*− 1.2*	*0.1442*	**−1.4**	**0.0411**
Metabolism - protein										
22	P22061	102	1.30E-05	9	28	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	**1.3**	**0.0046**	**1.5**	**0.0023**
106	Q96E11	75	6.10E-03	7	30	Ribosome recycling factor	**−1.6**	**0.0353**	**−1.4**	**0.0342**
Protein Chaperone										
104	O43301	103	1.00E-05	13	24	Heat shock 70 kDa protein 12A	**1.3**	**0.0250**	*1.3*	*0.0913*
62	P08107	98	2.70E-05	11	21	Heat shock 70 kDa protein 1	**−1.7**	**0.0417**	**−1.5**	**0.0106**

A

Protein #	SwissProt Accession	MASCOT MALDI-TOF significance scores[a] MASCOT score	Expect score	Hits	% Cov	Identification	Expression changes in ALCOHOLICS compared to CONTROLS[b] SFG Difference	SFG *P* values*	OC Difference	OC *P* values*
131	P08107	177	3.10E-13	13	36	Heat shock 70 kDa protein 1	*1.1*	*0.4296*	**1.4**	**0.0100**
147	P11142	67	3.60E-02	6	15	Heat shock 70 kDa protein 8	*− 1.1*	*0.2078*	**−1.4**	**0.0002**
36	P11142	141	1.50E-09	13	38	Heat shock 70 kDa protein 8	**−1.5**	**0.0199**	**−2.0**	**0.0002**
163	P11142	100	1.50E-05	10	29	Heat shock 70 kDa protein 8	*1.1*	*0.0667*	**1.3**	**0.0015**
31	P11142	162	9.70E-12	13	41	Heat shock 70 kDa protein 8	**1.2**	**0.0201**	**1.3**	**0.0011**
189	P17987	120	2.00E-07	15	28	T-complex protein 1 subunit alpha	*− 1.1*	*0.8123*	**−2.4**	**0.0364**
161	P54652	144	6.10E-10	16	28	Heat shock-related 70 kDa protein 2	*− 1.2*	*0.2096*	**−1.3**	**0.0099**
23	Q99497	96	5.60E-05	12	59	Protein DJ-1	**1.3**	**0.0395**	**1.3**	**0.0083**
Signal transduction										
152	P08758	125	4.80E-08	12	38	Annexin A5	**−1.5**	**0.0461**	*− 1.4*	*0.1038*
96	P09543	174	7.60E-13	15	32	2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase)	*− 1.4*	*0.4399*	**−1.9**	**0.0253**
156	P61981	102	9.70E-06	9	39	14-3-3 protein gamma	*− 1.2*	*0.1137*	**−1.3**	**0.0353**
145	P61981	88	2.20E-04	8	34	14-3-3 protein gamma	*− 1.2*	*0.4390*	**−1.5**	**0.0068**
146	P63096	67	3.00E-02	7	28	Guanine nucleotide-binding protein G(i), alpha-1 subunit	*1.0*	*0.7045*	**−1.5**	**0.0071**
Cytoskeleton										
128	P63261	72	9.50E-03	7	31	Gamma-actin	*− 1.5*	*0.3503*	**−2.2**	**0.0058**
15	Q16555	107	4.10E-06	13	25	Dihydropyrimidinase-related protein 2	*1.4*	*0.2218*	**1.7**	**0.0134**
100	Q16555	90	2.00E-04	9	25	Dihydropyrimidinase-related protein 2	*1.2*	*0.5463*	**1.8**	**0.0327**
Vesicle transport										
148	P61764	72	1.30E-02	9	18	Syntaxin--binding protein 1	*− 1.1*	*0.2555*	**−1.3**	**0.0081**
305	Q99719	162	9.70E-12	15	39	Septin-5	**−1.3**	**0.0319**	*1.0*	*0.9385*
61	Q05193	85	6.40E-04	10	14	Dynamin-1	**−2.1**	**0.0106**	*− 1.5*	*0.0590*
42	Q05193	139	1.90E-09	18	20	Dynamin-1	**−1.3**	**0.0504**	*− 1.1*	*0.5037*
37	Q05193	79	2.30E-03	13	18	Dynamin-1	**−1.5**	**0.0111**	**−1.5**	**0.0031**
40	Q05193	206	5.10E-16	23	25	Dynamin-1	**−1.4**	**0.0114**	*1.0*	*0.8514*
50	Q05193	90	1.90E-04	11	25	Dynamin-1	**−1.2**	**0.0072**	*− 1.0*	*0.6765*
46	Q05193	149	2.40E-10	15	14	Dynamin-1	*− 1.3*	*0.0554*	*− 1.0*	*0.6386*

A

Protein #	SwissProt Accession	MASCOT MALDI-TOF significance scores[a] MASCOT score	Expect score	Hits	% Cov	Identification	Expression changes in ALCOHOLICS compared to CONTROLS[b] SFG Difference	SFG *P* values*	OC Difference	OC *P* values*
58	Q05193	159	2.40E-11	16	17	Dynamin-1	*− 1.2*	*0.0575*	*− 1.1*	*0.1666*
39	Q05193	80	0.0022	12	16	Dynamin-1	*− 1.4*	*0.0891*	*− 1.3*	*0.0920*
55	Q05193	119	2.40E-07	12	22	Dynamin-1	*− 1.2*	*0.0893*	*− 1.1*	*0.2398*
270	Q05193	122	1.20E-07	12	21	Dynamin-1	*− 1.2*	*0.1220*	*− 1.2*	*0.1249*
54	Q05193	136	4.80E-09	13	22	Dynamin-1	*− 1.2*	*0.1589*	*− 1.1*	*0.4611*
52	Q05193	133	1.00E-08	19	23	Dynamin-1	*− 1.2*	*0.1994*	*− 1.1*	*0.3855*

B

Protein #	SwissProt Accession	MASCOT MALDI-TOF significance scores[a] MASCOT score	Expect score	Hits	% Cov	Identification	Expression changes in the SFG compared to OC[c] ALCOHOLICS Difference	ALCOHOLICS *P* values*	CONTROLS Difference	CONTROLS *P* values*
Metabolism - energy										
144	P11177	86	4.70E-04	11	36	Pyruvate dehydrogenase E1 component subunit beta	**1.2**	**0.0084**	*− 1.1*	*0.1561*
57	P12277	191	1.20E-14	15	54	Creatine kinase B-type	**−1.2**	**0.0041**	**−1.2**	**0.0004**
206	P00918	86	5.50E-04	7	35	Carbonic anhydrase 2	*− 1.2*	*0.1248*	**−1.3**	**0.0076**
126	P04075	87	3.40E-04	8	30	Fructose-bisphosphate aldolase A	*1.3*	*0.0770*	**1.7**	**0.0065**
160	P04075	164	6.10E-12	12	59	Fructose-bisphosphate aldolase A	*1.2*	*0.2154*	**1.6**	**0.0288**
86	P09972	142	9.70E-10	11	46	Fructose-bisphosphate aldolase C	*1.0*	*0.6549*	**1.5**	**0.0019**
205	P11216	182	1.20E-13	18	27	Glycogen phosphorylase, brain form	*− 1.2*	*0.1177*	**−1.3**	**0.0448**
Metabolism - oxidative phosphorylation										
122	P21281	154	6.10E-11	14	33	V-ATPase subunit B2	*1.1*	*0.1958*	**1.3**	**0.0029**
214	P06576	175	6.10E-13	17	58	ATP synthase subunit Beta	**−1.3**	**0.0380**	**−1.3**	**0.0396**
191	P25705	80	2.10E-03	7	18	ATP synthase subunit alpha	**−1.6**	**0.0281**	**−1.4**	**0.0376**
48	P25705	142	9.70E-10	14	32	ATP synthase subunit alpha	**−1.4**	**0.0376**	*− 1.2*	*0.1674*
203	P28331	159	1.90E-11	16	27	NADH-ubiquinone oxidoreductase 75 kDa subunit	**−1.6**	**0.0110**	*− 1.3*	*0.0973*
233	P30048	83	1.10E-03	7	45	Thioredoxin-dependent peroxide reductase	**−1.4**	**0.0473**	*− 1.2*	*0.0612*
Metabolism - protein										

B

Protein #	SwissProt Accession	MASCOT MALDI-TOF significance scores[a] MASCOT score	Expect score	Hits	% Cov	Identification	Expression changes in the SFG compared to OC[c] ALCOHOLICS Difference	ALCOHOLICS *P* values*	CONTROLS Difference	CONTROLS *P* values*
241	P00505	214	6.10E-17	17	38	Aspartate aminotransferase	**−1.4**	**0.0278**	**−1.4**	**0.0208**
22	P22061	102	1.30E-05	9	28	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	**1.3**	**0.0171**	**1.5**	**0.0101**
Protein Chaperone										
104	O43301	103	1.00E-05	13	24	Heat shock 70 kDa protein 12A	**1.3**	**0.0139**	**1.3**	**0.0455**
89	O43301	84	8.00E-04	11	23	Heat shock 70 kDa protein 12A	**1.7**	**0.0189**	**2.6**	**0.0025**
62	P08107	98	2.70E-05	11	21	Heat shock 70 kDa protein 1	*− 1.7*	*0.0760*	**−1.5**	**0.0237**
131	P08107	177	3.10E-13	13	36	Heat shock 70 kDa protein 1	**−1.3**	**0.0343**	*1.0*	*0.7647*
260	P11021	184	6.10E-14	17	31	Heat shock 70 kDa protein 5	*− 1.2*	*0.0763*	**−1.4**	**0.0339**
147	P11142	67	3.60E-02	6	15	Heat shock 70 kDa protein 8	*− 1.0*	*0.6970*	**−1.3**	**0.0012**
36	P11142	141	1.50E-09	13	38	Heat shock 70 kDa protein 8	*− 1.1*	*0.1063*	**−1.5**	**0.0002**
163	P11142	100	1.50E-05	10	29	Heat shock 70 kDa protein 8	*1.1*	*0.0739*	**1.3**	**0.0028**
189	P17987	120	2.00E-07	15	28	T-complex protein 1 subunit alpha	*1.1*	*0.8770*	**−2.0**	**0.0237**
161	P54652	144	6.10E-10	16	28	Heat shock-related 70 kDa protein 2	**−1.3**	**0.0283**	**−1.5**	**0.0017**
Signal transduction										
337	P09471	116	3.90E-07	8	29	Guanine nucleotide-binding protein G(o) subunit alpha	**1.6**	**0.0009**	**1.3**	**0.0055**
96	P09543	174	7.60E-13	15	32	2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase)	*− 1.1*	*0.5540*	**−1.5**	**0.0041**
145	P61981	88	2.20E-04	8	34	14-3-3 protein gamma	*− 1.1*	*0.6357*	**−1.3**	**0.0185**
166	P63104	94	6.70E-05	9	36	14-3-3 protein zeta/delta	**1.2**	**0.0043**	*− 1.1*	*0.2424*
Cytoskeleton										
120	Q13561	66	3.80E-02	8	30	Dynactin subunit 2	**−1.2**	**0.0120**	**−1.2**	**0.0148**
100	Q16555	90	2.00E-04	9	25	Dihydropyrimidinase-related protein 2	*1.1*	*0.6632*	**1.6**	**0.0511**
Vesicle transport										
307	P07196	124	6.10E-08	14	30	Neurofilament light polypeptide	**−2.3**	**0.0454**	*1.0*	*0.9391*
148	P61764	72	1.30E-02	9	18	Syntaxin-binding protein 1	**1.3**	**0.0043**	*1.1*	*0.1257*
240	Q9H115	178	2.40E-13	14	69	Beta-soluble NSF attachment protein	**1.3**	**0.0191**	*1.1*	*0.2502*
305	Q99719	162	9.70E-12	15	39	Septin-5	*1.1*	*0.4336*	**1.4**	**0.0256**
39	Q05193	80	2.20E-03	12	16	Dynamin-1	**−1.4**	**0.0343**	**−1.3**	**0.0354**
40	Q05193	206	5.10E-16	23	25	Dynamin-1	*1.1*	*0.6045*	**1.5**	**0.0347**
50	Q05193	90	1.90E-04	11	25	Dynamin-1	*1.1*	*0.4643*	**1.3**	**0.0089**

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B

Protein #	SwissProt Accession	MASCOT MALDI-TOF significance scores[a]				Identification	Expression changes in the SFG compared to OC[c]			
							ALCOHOLICS		CONTROLS	
		MASCOT score	Expect score	Hits	% Cov		Difference	*P* values*	Difference	*P* values*
37	Q05193	79	2.30E-03	13	18	Dynamin-1	*− 1.2*	*0.1681*	*− 1.2*	*0.0665*
46	Q05193	149	2.40E-10	15	14	Dynamin-1	*− 1.0*	*0.6218*	*1.2*	*0.0904*
42	Q05193	139	1.90E-09	18	20	Dynamin-1	*1.0*	*0.7218*	*1.2*	*0.1337*
58	Q05193	159	2.40E-11	16	17	Dynamin-1	*− 1.2*	*0.0588*	*− 1.1*	*0.1517*
61	Q05193	85	6.40E-04	10	14	Dynamin-1	*− 1.2*	*0.5838*	*1.2*	*0.2636*
55	Q05193	119	2.40E-07	12	22	Dynamin-1	*− 1.1*	*0.1582*	*− 1.1*	*0.3496*
52	Q05193	133	1.00E-08	19	23	Dynamin-1	*− 1.0*	*0.8587*	*1.1*	*0.3854*
270	Q05193	122	1.20E-07	12	21	Dynamin-1	*− 1.1*	*0.4732*	*− 1.1*	*0.4799*
54	Q05193	136	4.80E-09	13	22	Dynamin-1	*− 1.1*	*0.2593*	*1.0*	*0.7758*

[a] MALDI-TOF significance scores: MASCOT score, a measure of the statistical significance of a match generated during the MASCOT search. Protein scores greater than the database score (NCBInr *Homo sapiens* score = 64) are significant. Expect score, the number of times you would expect to get this score, or better, by chance; Hits, the number of experimental peptides found to match the protein in the database; % Cov, the amount that the matched peptides match the identified protein sequence.

[*] *P* values generated by Newman-Keuls post-hoc correction of ANCOVA values.

[b] A positive expression change indicates a protein that is higher in alcoholics than in controls.

[c] A positive expression change indicates a protein that is higher in the SFG than in the OC.