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PRENATAL ALCOHOL EXPOSURE ALTERS THE CEREBRAL CORTEX PROTEOME IN WEANLING RATS

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Abstract

Maternal consumption of alcohol during pregnancy impairs neurodevelopment in offspring. Utilizing a rodent model of continuous moderate dose alcohol exposure throughout gestation [gestation day 1 (GD1)-GD22; BAC ~70 mg/dL], the impact of developmental alcohol exposure on juvenile cerebral cortex protein abundances was determined. At weaning, cerebral cortex tissue was collected from pups for 2D SDS-PAGE based proteome analysis with statistical analysis by Partial Least Squares-Discriminant Analysis (PLS-DA). Gestational alcohol exposure increased the abundance of post-translationally modified forms of cytoskeletal proteins and the abundance of proteins within the small molecule biochemistry (includes glucose metabolism) pathway and proteasome processing pathways though ubiquitin conjugating enzymes and chaperones were decreased in abundance. We interpret the findings of this study as an indication that prenatal alcohol exposure, in the absence of low birth weight but with delayed neurodevelopment, leads to a persistent systemic metabolic dysregulation with attendant impaired chaperoning/protein processing.

Keywords

prenatal alcohol exposure; FAS; proteome; cortex

1. INTRODUCTION

Originally described in 1973, fetal alcohol syndrome (FAS) is characterized by prenatal/postnatal growth deficiencies, central nervous system dysfunction, and craniofacial defects [1, 2]. Recognition of a subclinical spectrum of effects associated with prenatal alcohol exposure [fetal alcohol spectrum disorders (FASD)] occurred within the following decades with the finding that there is no safe threshold for alcohol exposure during pregnancy [3-6]. Accordingly, the CDC recommends that reproductive age women restrict consumption of alcohol to no more than seven alcohol containing drinks per week and no more than three drinks per occasion [7]. Despite prominent public health campaigns to raise awareness of the dangers of alcohol consumption during pregnancy, a significant number of expectant mothers continue to consume alcohol at low to moderate levels [8-10]. It is estimated that

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between 1-5 percent of births in the US may be impacted by FASD [11, 12] with similar findings in Europe and Asia [13-18].

Binge consumption of alcohol during pregnancy is tightly linked to the neurocognitive defects associated with FAS, while the impact of lower levels of maternal alcohol consumption on neurodevelopment is somewhat more diffuse. In rodent models, gestational alcohol exposure is associated with a host of neurodevelopmental delays including delayed reflex ontogeny, anxiety, spatial learning deficits, fear response, and delayed neuromuscular development [19-27]. The cognitive abnormalities point to the impact of prenatal alcohol exposure on both the cerebral cortex (higher order thought) and hippocampus (memory and emotion). Persistent alterations in brain region specific gene expression of neurotransmitters, transcription factors, cell proliferation, and cell survival have been found in studies utilizing a variety of acute alcohol exposure regimes within rodent models [28-32]. In the context of low to moderate level alcohol exposure spanning murine neurodevelopment (~100 mg/dL), adult whole brain gene expression profiling identified disruption of pathways related to neurodevelopment, RNA and DNA regulation, and small molecule metabolism [33].

A variety of environmental co-exposures during gestation including stress and maternal nutrition may contribute to the subclinical manifestations of FAS/FASD [34-41]. In the only study to date related to protein expression in the brain following dietary modulation concurrent with high dose alcohol exposure during gestation (GD18; whole brain), folic acid supplementation was found to partially alleviate the alcohol induced suppression of proteins involved in energy production and metabolism as well as protein translation, folding and signaling though the inclusion of a maternal pair-fed control was lacking and thus the impact of maternal caloric restriction underlying the alcohol model on fetus brain protein expression could not be established [42]. Together with the dysregulation of gene expression data, this study further supports the hypothesis that prenatal alcohol exposure induces persistent alterations in neurobiology and further that diet influences pathogenesis.

In the current study, we investigated the pattern of persistent moderate level prenatal alcohol exposure-induced proteome alterations within the cerebral cortex of juvenile Sprague Dawley offspring compared to offspring from dams with matched caloric consumption during gestation. A typical Western diet is high in carbohydrates and fat. The present study utilized Everclear, an inexpensive and common source of alcohol for young adults, as the alcohol source (36% of calories) within a Leiber-DeCarli dietary matrix that includes 25% of calories from protein, and 12% of calories from fat (olive, safflower, and corn oil) with a total carbohydrate consumption as 63% of diet [43]. This study examines cerebral cortex protein expression in offspring at weaning, 3 weeks past cessation of alcohol consumption, in a model that exhibits delayed neurodevelopment as measured by self-righting reflex ontogeny.

2. MATERIALS AND METHODS

2.1 Animal Exposure Model

Adult Sprague Dawley rats aged 8-12 weeks were purchased from Charles River Labs. Animals were housed and maintained in the University of Louisville Research Resources Center, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Animals were maintained in a controlled temperature/humidity environment with a 12 hour light/dark cycle and free access to a modified Leiber-DeCarli liquid diet (LD'82 Pregnant, High Protein; Bioserve, Frenchtown, NJ) control diet. Sham liquid diet without maltodextrin added was introduced with unrestricted access 1 week prior to mating. Following diet acclimatization, adult male and nulliparous female Sprague Dawley rats were housed together overnight with the presence of a seminal plug the

following morning designated as gestational day (GD) 0. Alcohol administration (EtOH group; n=10 for proteome studies) as 36% of the dietary calories or maltodextrin (Sham group; pair-fed to EtOH group; n=8 for proteome studies) as 36% of dietary calories (substituted for alcohol) began GD0 and continued until the day of birth at which time dams were switched to solid Purina lab diet 5001 for maintenance. Blood alcohol levels were measured during the 8-10 am window on GD 15 with a finding of BAC of 70 mg/dL. At PD24-26, pups were euthanized by carbon dioxide asphyxiation, cerebral cortex dissected on ice, and tissue stored at -80°C until analysis.

2.2. Pup Development and Self-Righting Reflex Measurement

From PD1-21, pup weights (litter as statistical unit; total of 15 litters per group) were collected daily. Statistical analysis included logistic regression comparing slope and intercept of the lines as well as elevation. The self-righting reflex was tested on PD2-7 by allowing the supine pup to attempt to right within 30 seconds. A positive test consisted of all four paws on the ground within the 30 seconds with a yes/no designated as outcome. The percent success for each day was calculated for each litter and then averaged across litters within each group. Student's t-test for individual day outcomes was used for comparison with $p < 0.05$ considered significant [44].

2.3 2D-SDS-PAGE

Cortex tissue (~ 0.05 g tissue wet weight; 8 Sham and 10 EtOH liver samples from a single male per litter euthanized at 3 weeks of age) was homogenized in 0.5 ml sample preparation buffer [7M urea, 2M thiourea, 40mM dithiothreitol (DTT)] with protein concentration for each sample determined by Bradford Assay [45]. Three hundred and fifty micrograms of protein in rehydration buffer (8M urea, 2% CHAPS, 2 μl IPG buffer pH 3-10, 2.5 mg/ml DTT, 0.002% bromophenol blue) was applied to IPGphor Drystrips (Nonlinear, 3-10NL, 180 mm $\times$ 3 mm $\times$ 0.5 mm, GE Healthcare, Piscataway, NJ). First dimension separation by isoelectric focusing at 22,000 Volt hours (Vhrs) was performed with a hold at 100 Volts until further processing. The IEF strips were stored at -80°C for 1 hr followed by: 1) equilibration for 30 minutes in reducing buffer (6M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue with 3.5 mg/ml DTT) and 2) equilibration in alkylating buffer (same buffer with 45 mg/ml iodoacetamide instead of DTT) for an additional 30 minutes. Second dimension SDS-PAGE separation (25cm $\times$ 20.5cm 15% polyacrylamide gels) was performed overnight (18 hrs; 100V). Protein spots were visualized by Colloidal Coomassie Blue G-250 (3 day staining) followed by water washes until a clear background was evident [46].

2.4 Image Analysis

Gel images were collected with an Epson Expression 10000 XL scanner. Densitometric analysis was performed with Progenesis SameSpots software (Nonlinear Dynamics; New Castle-on-Tyne, UK). Protein spots were detected automatically with manual adjustment as necessary for accuracy. For each protein spot, intensity was measured, background subtracted, and individual spot density normalized by total pixel density of each gel. Spots with average normalized pixel depth 1000 relative abundance units and non-normalized areas with pixel depth below 1000 were removed as noise.

2.5 Statistical Analysis

Data were analyzed using PASW Statistics 18 software. Partial Least Squares-Discriminant Analysis (PLS-DA) modeling of variance between groups with Variable Importance in Projection (VIP) rankings was used to identify protein spot features whose normalized abundance determined the differences between groups [47, 48]. Sequential removal of top

ranked protein spots (inverse recursive feature elimination) was performed until the variance between groups was eliminated. The features with the highest ranking were eliminated in 25 feature groups until the plot separation was eliminated or the empiric threshold value of 1.25 for VIP score was passed. Due to the limited number of biological replicates per group (n=8 for SHAM and n=10 for EtOH), the dataset was not split into a test and validation set [46, 49].

2.6 Mass Spectrometry-Based Protein Spot Identification

Protein spots were excised and destained with 50% ethanol in 50 mM NH_4HCO_3 for a minimum of 5 washes. Proteins were digested with 10 ng/ μl trypsin in 40mM NH_4HCO_3 at room temperature for approximately 18 hrs. Peptides were eluted in 50% ACN/5% formic acid followed by 95% ACN/5% formic acid and stored at -20°C until analysis. The mass to charge ratio of peptides was determined by direct inject LTQ/FT-ICR-MS/MS with collision induced dissociation for structural feature identification. Peptide identification was performed with the Mascot (Matrix Sciences v 2.2.2) search algorithm utilizing the NCBI Inr (with decoy) database (updated June 1, 2010). Search parameters included: mammalian class, 2 missed cleavages, carbamidomethyl C variable modification, enzyme trypsin/P, and an allowed peptide charge of 1^+ , 2^+ , or 3^+ . Positive protein identification required a total MOWSE absolute probability entire protein score ≥ 100 composed of a minimum of two peptides with individual scores MOWSE absolute probability scores ≥ 50 [50].

3. RESULTS

3.1 Exposure Conditions and Outcomes

As shown in Figure 1, the sustained moderate blood alcohol levels (~ 70 mg/dL) on a high fat dietary background attained in this study did not result in a low birth weight phenotype. Neurodevelopment as measured by self-righting reflex was delayed in acquisition in the EtOH group as compared to the Pair-Fed Sham exposure group. The percent of successful tests (litter as the statistical unit) is shown with a deficit in motor skill evident on PD2 through PD6 though maturation of the reflex does develop by PD8. Independent significance in the delay of acquisition in offspring exposed prenatally to alcohol was present on PD2 ($p=0.03$) and PD3 ($p=0.01$). This finding indicates a delay in maturation of the response by approximately 1 day. This supports findings by Thomas and others that subtle motor development impairment persists past the withdrawal of alcohol exposure [44].

3.2 PLS-DA modeling

As shown in Figure 2, a ranked order of importance (Variable Import in Projection: VIP) of protein spot densities was utilized in defining the differences between groups after building a multivariate inverse least squares discriminant model to classify sample groupings. Noise (pixel depth less than 1000 as describe in Section 2.4) was removed from the dataset prior to PLS-DA modeling leaving 1048 protein spots for inclusion. Iterative PLS-DA models with recursive feature elimination were generated encompassing normalized spot abundances of all proteins. When abundances of all protein spots were included, the first latent factor described approximately 69% of variance between groups with the second latent factor describing an additional 22% of variance for a total of 92% description of variance. Approximately 75 protein spots described the variance between the groups. When the top two latent factors were plotted, the separation between the groups was evident.

3.3 Proteome Profiles

As shown in Figure 3, the cerebral cortex protein spot patterns spanned an isoelectric focusing range of 3 to 10, with the acidic proteins on the left side of the gel image, the basic

proteins on the right, and molecular weights descending from above 60 kDa to approximately 12 kDa. The protein spot patterns of gels from the SHAM and EtOH groups were similar without the presence/absence of spots between groups. Varying pixel depths (spot abundances) were the dominant difference between groups. Of the 75 protein spots ranked as of greatest import in the PLS-DA modeling, 40 spots were identified. The blue numbers (n=22) on the gel were protein spots increased in abundance while the red numbers (n=20) were protein spots decreased in abundance. Protein identifications are listed in Table 1 (increased abundance) and Table 2 (decreased abundance).

3.4 Cytoskeletal proteins

Several protein spots increased in abundance were identified as cytoskeletal (and associated) proteins including **Spots 1, 2, 3, 4, 13, 18, and 20** that included Actin, Tubulin, and Actinin. These protein spots were generally clustered in the central region of the gel shown in Figure 3. The proportionally low abundance relative to the total spot density of the gel (represents less than 10 percent of the total) as well as the previous reports of location of dominant tubulin spots (clustered in the acidic zone at approximately 50 kDa) indicates that these spots are post-translationally modified or cleavage products of the identified proteins. **Spot 32** (decreased) was also identified as a truncated form of Actin and again likely reflects an alteration in post-translational processing of cytoskeletal proteins in the cerebral cortex of offspring exposed to continuous moderate level alcohol exposure throughout gestation.

3.5 Small Molecule Metabolism

Several enzymes related to glucose, pyruvate, and amino acid metabolic processes were increased in abundance in the cerebral cortex of weaning offspring exposed to continuous moderate levels of alcohol during gestation. **Spots 6, 7, and 14** are enzymes of the gluconeogenic/glycolytic pathway. **Spot 9** was identified as Pyruvate Dehydrogenase E1 subunit 1, a part of the pyruvate dehydrogenase complex that converts pyruvate to acetyl-CoA prior to utilization in the TCA cycle. **Spots 10 and 22** were identified as cytosolic Aspartate Aminotransferase, an enzyme important in the synthesis of glutamate within the cerebellum and possibly other brain subregions. RBL-NDP kinase 18kDa subunit (p18) (**Spot 5**) is involved in nucleoside processing.

3.6 Protein chaperoning/degradation/processing

Proteasome alpha 4 subunit (**Spot 8**), Chaperonin containing TCP1, subunit 2 (beta) (**Spot 17**), Psmd11 protein (**Spot 15**), and N-acetylneuraminic acid synthase (**Spot 19**) were *increased* in abundance in the EtOH group. Proteins from **Spot 8 and 15** are directly involved in proteasome mediated degradation of ubiquitinated proteins and imply an increase in protein turnover. Chaperonin containing TCP1, subunit 2 (beta) (**Spot 17**) is involved in actin and tubulin folding while N-acetylneuraminic acid synthase (**Spot 19**) mediates the formation of sialic acid, a prevalent posttranslational modification of glycoproteins. The Ubiquitin-conjugating enzyme E2N (**Spot 41**), an enzyme responsible for attachment of ubiquitin to proteins targeted for degradation by the proteasome, was *decreased* in abundance in the cerebral cortex of the EtOH group. Ubiquitin carboxyl-terminal esterase L1 (**Spot 38**), a dual function enzyme that both removes ubiquitin from proteins targeted for degradation as well as creating polymeric ubiquitin chains for attachment to proteins to signal proteasome dependent degradation machinery, was also *decreased* in abundance in the cerebral cortex of the EtOH group.

Heat shock 70 kDa protein 4 (**Spot 25**; chaperone) and Peroxiredoxin 2 (**Spots 27 and 34**; prevents inappropriate protein thiol disulphide linkages) protein spot abundances were *decreased* in the cerebral cortex of offspring prenatally exposed to alcohol. Calreticulin

(**Spot 28**), which binds misfolded proteins in the endoplasmic reticulum, was also decreased in abundance in the EtOH group.

3.7 Mitochondrial Function

ATP synthase alpha subunit precursor (**Spot 33**) and NADH dehydrogenase ubiquinone flavoprotein 2 precursor (**Spot 36**) are a part of the mitochondrial respiratory chain while Voltage-dependent anion channel 1 (**Spot 42**) is a mitochondrial membrane channel--all of which were decreased in abundance in the cerebral cortex of offspring exposed prenatally to EtOH.

3.8 Vesicle/Membrane Fusion/Cell Signaling

Protein spots identified as Valosin-containing protein (**Spot 31**) and N-ethylmaleimide-sensitive factor attachment protein, alpha (**Spot 39**) were decreased in abundance in the EtOH group cerebral cortex, indicating a possible inhibition of vesicle/membrane fusion. Guanine nucleotide-binding protein alpha subunit (**Spot 29**), Myotrophin (**Spot 30**; Nf B dependent) and Rho GDP dissociation inhibitor (GDI) alpha (**Spot 37**) were decreased in abundance in the cerebral cortex of the offspring exposed prenatally to EtOH.

3.9 Calcium Dependent/Sensing

Protein spots identified as Ncald protein (**Spot 24**; neuronal calcium sensor (NCS) family) and Calreticulin (**Spot 28**; binds misfolded proteins in the endoplasmic reticulum) were decreased in abundance in the cerebral cortex of the EtOH group.

4. DISCUSSION

Maternal alcohol consumption is detrimental to offspring health with the most common outcomes including low birth weight, developmental delays, and cognitive impairment [3-6]. The original Leiber-Decarli Diet [51] induced liver injury, hepatic fibrosis, and steatosis when fed for 8 weeks though the type of dietary fat was an important regulator of the molecular pathogenesis [52, 53]. In the current study, a modified diet derived to support pregnancy and to minimize liver damage to the dam was utilized [43]. Neurodevelopmental delays, fetal hematopoietic cell development and the development of the immune system were compromised in studies utilizing similar diets [28, 54-61]. A delay in the self-righting reflex was documented in the current study of cerebral cortex proteome alterations. This metric is a measure of reflex development primarily centered in the motor cortex that has been widely described as impacted by high dosages of prenatal alcohol exposure [62-64]. Typically, assessment of higher order thought processes controlled by the cerebral cortex are completed at full maturation rather than at weaning. Though not descriptive of the function of the motor cortex at the time of tissue collection for proteome profiling, the mild neurodevelopmental delay observed in offspring exposed to moderate dosages of alcohol during gestation allows comparison of these findings across a variety of exposure paradigms.

We examined whether gestational alcohol exposure induced impairment of neurodevelopment resulted in persistent alterations of protein expression in the cerebral cortex of juvenile offspring. In postmortem clinical studies, the impact of adult alcoholism on white and grey matter specific protein expression profiles found abnormalities in thiamine-dependent metabolic enzymes suggesting a reduction in thiamine availability [65-67]. This is unsurprising given the thiamine deficiency of alcoholics [68, 69]. In particular, pyruvate dehydrogenase (E1) and transketolase were found to be decreased in the dorsolateral prefrontal cortex, the superior frontal cortex, and the cerebellar vermis of active alcoholics while dihydrolipamide dehydrogenase was decreased in the cerebellar

vermis only [66, 67, 70, 71]. The pyruvate dehydrogenase complex is composed of multiple copies of pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) which together catalyze the conversion of pyruvate to acetyl-CoA and carbon dioxide. Pyruvate decarboxylation links glycolysis to the citric acid cycle in support of cellular respiration.

In contrast, the current study found an increase in expression of pyruvate dehydrogenase (E1) in the cerebral cortex of juvenile offspring exposed *in utero* to moderate levels of alcohol. While it is possible that a mild thiamine deficiency occurred during alcohol exposure due to a slight reduction in feeding behavior, this was matched in the pair-fed group and offspring were maintained from birth until weaning on a thiamine sufficient diet with no difference in consumption of diet between the groups. In light of the lack of differential thiamine availability between groups during postnatal development, and the lack of impact on multiple other proteins associated with thiamine availability/processing, we interpret our finding of increased expression of pyruvate dehydrogenase (E1) as indicative of an increased call for substrate in support of cellular respiration.

A significant increase in expression of enzymes within the glycolysis and glycogen storage pathways of synaptosomes of the superior frontal gyrus and the occipital cortex of alcoholics was previously observed [72]. In the current study, increased abundance of cytosolic malate dehydrogenase (production of pyruvate or oxaloacetate), triosephosphate isomerase 1 (glycolysis/gluconeogenesis), and glyceraldehyde-3-phosphate dehydrogenase (glycolysis/gluconeogenesis) were also found in the cerebral cortex of juvenile offspring prenatally exposed to alcohol. Together with the increased expression of pyruvate dehydrogenase (E1), the increased abundance of enzymes involved in glucose metabolism points to an enduring stimulation of prenatal alcohol exposure, long after cessation, on glucose utilization within grey matter. We interpret this finding as supportive of the generalized hypothesis that neurodevelopment is delayed including the formation of specific sub tissue functional regions thus energy utilization is increased in the juvenile period to support cellular population establishment past the normal timing similar to the phenomena of “catch-up” growth but rather termed catch-up functional establishment.

In contrast, decreased glucose metabolism was identified in the cerebellar vermis of patients with alcoholic cerebellar degeneration [73] as well as decreased expression of enzymes in glycolysis and the tricarboxylic acid cycle of both the cerebellar vermis [66]. This may point to a differential impact of alcohol on white matter versus grey matter, but more likely reflects advanced neurodegeneration including cell death typified by chronic alcohol abuse in adulthood rather than neurodevelopmental impairment due to prenatal alcohol exposure. Our study did not include an examination of both grey and white matter and therefore does not address regional variation in the impact of developmental alcohol exposure on proteome profiles.

In the current study, an *increase* in abundance of enzymes that target proteins for degradation by the proteasome was found but a *decrease* in proteins responsible for ubiquitin attachment/removal and protein stabilization within the endoplasmic reticulum were found. The impairment of turnover of damaged proteins represents a reduced capacity to effectively counter aberrant protein post-translational modifications such as inappropriate disulphide bridge formation or acetylation that alters either function or form of the workhorses of the cell. Though we did not assess the oxidative status of the cerebral cortex in juvenile offspring following gestational alcohol exposure, oxidative stress is a feature of both chronic and acute alcohol exposure. For example, an increase in abundance of GRP-75, calreticulin and protein disulfide isomerase in the cerebellar vermis of alcoholics has been reported that are indicative of the increased oxidative stress accompanying alcohol consumption [66]. We

interpret our findings as indicative of an adaptive response to persistent oxidative stress resulting from developmental alcohol exposure during neurogenesis that likely impairs the capacity to repair a subsequent oxidative challenge.

In summary, we have identified proteins within the cerebral cortex of juvenile offspring that are altered by prenatal alcohol exposure. Though energy metabolism is affected, it is unlikely that persistent thiamine deficiency is responsible. We interpret the findings of this study as an indication that prenatal alcohol exposure, in the absence of low birth weight but with delayed cortex neurodevelopment, leads to a persistent metabolic dysregulation with attendant impaired chaperoning/protein processing. Future studies examining the continuity of impact on metabolic pathways post-cessation of exposure are of great interest in understanding the association of prenatal alcohol exposure induced cognitive impairment.

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Highlights

- Prenatal Alcohol Exposure Alters Cerebral Cortex Small Molecule Metabolism
- Cerebral Cortex Energy Regulation Enzymes Altered By Prenatal Alcohol Exposure
- Cerebral Cortex Proteome Profile Altered By Prenatal Alcohol Exposure.

Figure 1A. Prenatal Moderate Dose Ethanol Exposure Did Not Alter Birth Weight
Moderate alcohol exposure throughout gestation did not impact offspring weights (litter as unit).

Figure 1B. Delayed Self-righting Reflex Acquisition Found in Neonatal Offspring Exposed to Alcohol In Utero

A delay in maturation of the self-righting reflex was observed in neonatal offspring exposed to alcohol throughout gestation. A significant decrease in acquisition of this skill was evident on PD2 and PD3.

Figure 2. The Cortex Proteome Profiles Are Altered in Offspring Exposed Prenatally to Ethanol
The top 75 spots according to Variable Importance for Projection (VIP) ranking are included. A separation of the groups is evident when the first two latent factors derived from the PLS-DA model are graphed.

Figure 3. Cerebral Cortex Proteome Profiles Altered by Prenatal Ethanol Exposure

The cerebral cortex proteins with altered abundance following prenatal ethanol exposure are circled and numbered. Refer to Table 1 (increased) and Table 2 (decreased) for protein identifications.

Identification of Protein Spots Increased in Abundance in the Cerebral Cortex at PD21 Following Prenatal EtOH exposure

Table 1

VIP Ranking	Figure #	ID	MOWSE Score	# Peptides	% Coverage	Mw (kDa)
2.4	1	gi 224839 tubulin T beta15	127	6	20	49.9
2.3	2	gi 6678467 Tubulin, alpha 4	372	9	27	49.9
2.3	3	gi 6755901 Tubulin, alpha 1	143	5	12	50.1
2.3	4	gi 1142640 alpha Actinin	123	4	5	102.5
2.3	5	gi 206580 RBL-NDP kinase 18kDa subunit (p18)	200	5	39	17.4
2.2	6	gi 387129 Cytosolic malate dehydrogenase	149	6	22	36.5
2.2	7	gi 117935064 Triosephosphate isomerase 1	737	13	73	26.8
2.1	8	gi 6755196 Proteasome alpha 4 subunit	86	3	10	29.5
2.0	9	gi 57657 Pyruvate dehydrogenase E1 alpha form 1 subunit	79	2	6	43.2
2.0	10	gi 220684 Cytosolic aspartate aminotransferase	677	14	49	46.4
2.0	11	gi 203658 Cu-Zn superoxide dismutase	190	4	32	15.7
2.0	12	gi 114145722 Hypothetical protein LOC302247	74	3	12	33.8
1.9	13	gi 809561 Gamma-actin	125	5	18	41
1.9	14	gi 56188 Glyceraldehyde 3-phosphate-dehydrogenase	90	3	12	35.8
1.9	15	gi 33585718 Psmd11 protein	236	8	24	46.9
1.8	16	gi 13928886 Mitogen-activated protein kinase kinase 1	100	3	10	43.5
1.8	17	gi 54400730 Chaperonin containing TCP1, subunit 2 (beta)	563	13	37	57.4
1.8	18	gi 6678465 Tubulin, alpha 1a	102	4	13	49.9
1.8	19	gi 164663874 N-acetylneuraminic acid synthase	133	5	24	40
1.8	20	gi 223556 Tubulin alpha	241	7	23	50.2
1.8	21	gi 6714522 Dihydropyrimidinase-related protein	298	9	20	61.4
1.8	22	gi 220684 Cytosolic aspartate aminotransferase	677	14	49	46.4

Identification of Protein Spots Decreased in Abundance in the Cerebral Cortex at PD21 Following Prenatal EtOH exposure

Table 2

VIP Ranking	Figure #	ID	MOWSE Score	# Peptides	% Coverage	Mw (kDa)
2.3	23	gi 8393693 Laminin receptor 1	171	6	24	32.8
2.3	24	gi 15029877 Ncald protein	317	8	47	22.2
2.1	25	gi 2495342 Heat shock 70 kDa protein 4	69	2	3	94.1
2.1	26	gi 2970691 Thioredoxin-related protein	110	3	19	32.2
2.0	27	gi 149049177 Heme binding protein 1 (predicted), isoform CRA_a	175	5	42	21.1
		gi 34849738 Peroxiredoxin 2	111	3	18	21.8
2.0	28	gi 11693172 Calreticulin	533	16	47	48
1.9	29	gi 2044444 Guanine nucleotide-binding protein alpha subunit	109	4	13	35.4
1.9	30	gi 6679961 Myotrophin	153	3	48	12.8
1.9	31	gi 17865351 Valosin-containing protein	448	14	25	89.3
1.9	32	gi 49868 put. Beta-actin (aa 27-375)	624	15	60	39.2
1.9	33	gi 203055 ATP synthase alpha subunit precursor	472	12	28	58.8
1.9	34	gi 34849738 Peroxiredoxin 2	340	8	47	21.8
1.9	35	gi 3959370 Beta-2 globin	298	7	49	16
1.8	36	gi 51092268 NADH dehydrogenase ubiquinone flavoprotein 2 precursor	219	6	32	27.4
1.8	37	gi 13435747 Rho GDP dissociation inhibitor (GDI) alpha	249	7	48	23.4
1.8	38	gi 61098212 Ubiquitin carboxyl-terminal esterase L1	371	7	48	24.8
1.8	39	gi 18034791 N-ethylmaleimide-sensitive factor attachment protein, alpha	375	9	36	33.2
1.8	40	gi 6754012 Guanine nucleotide binding protein, alpha o isoform A	90	4	15	40.1
1.5	41	gi 16758810 Ubiquitin-conjugating enzyme E2N	255	7	51	17.1
1.7	42	gi 6755963 Voltage-dependent anion channel 1	368	9	46	32.3