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Ethanol inhibition of aspartyl-asparaginyl- β -hydroxylase in fetal alcohol spectrum disorder: Potential link to the impairments in central nervous system neuronal migration

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Abstract

Fetal alcohol spectrum disorder (FASD) is caused by prenatal exposure to alcohol and associated with hypoplasia and impaired neuronal migration in the cerebellum. Neuronal survival and motility are stimulated by insulin and insulin-like growth factor (IGF), whose signaling pathways are major targets of ethanol neurotoxicity. To better understand the mechanisms of ethanol-impaired neuronal migration during development, we examined the effects of chronic gestational exposure to ethanol on aspartyl (asparaginyl)- β -hydroxylase (AAH) expression, because AAH is regulated by insulin/IGF and mediates neuronal motility. Pregnant Long—Evans rats were pair-fed isocaloric liquid diets containing 0, 8, 18, 26, or 37% ethanol by caloric content from gestation day 6 through delivery. Cerebella harvested from postnatal day 1 pups were used to examine AAH expression in tissue, and neuronal motility in Boyden chamber assays. We also used cerebellar neuron cultures to examine the effects of ethanol on insulin/IGF—stimulated AAH expression, and assess the role of GSK-3 β —mediated phosphorylation on AAH protein levels. Chronic gestational exposure to ethanol caused dose-dependent impairments in neuronal migration and corresponding reductions in AAH protein expression in developing cerebella. In addition, prenatal ethanol exposure inhibited insulin and IGF-I—stimulated directional motility in isolated cerebellar granule neurons. Ethanol-treated neuronal cultures (50 mM $\times$ 96 h) also had reduced levels of AAH protein. Mechanistically, we showed that AAH protein could be phosphorylated on Ser residues by GSK-3 β , and that chemical inhibition of GSK-3 β and/or global Caspases increases AAH protein in both control- and ethanol-exposed cells. Ethanol-impaired neuronal migration in FASD is associated with reduced AAH expression. Because ethanol increases the activities of both GSK-3 β and Caspases, the inhibitory effect of ethanol on neuronal migration could be mediated by increased GSK-3 β phosphorylation and Caspase degradation of AAH protein.

Keywords

Aspartyl (asparaginyl)- β -hydroxylase; Fetal alcohol syndrome; Neuronal migration; Insulin; Central nervous system; Glycogen synthase kinase 3 β

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Introduction

Fetal alcohol spectrum disorders (FASD) are caused by maternal alcohol consumption during pregnancy (Riley and McGee, 2005) and are associated with central nervous system (CNS) malformations and mental retardation (Abel, 1984; Danis et al., 1981), cognitive impairments and behavioral dysfunctions that vary in severity with levels, duration, and timing of prenatal alcohol exposure (Aronson et al., 1997; Coffin et al., 2005; Goodlett and Eilers, 1997; Goodlett and Lundahl, 1996; Hausknecht et al., 2005; O'Malley and Nanson, 2002; Scahill and Schwab-Stone, 2000). Prominent CNS abnormalities observed in FASD include cerebellar hypoplasia and impaired neuronal migration (Archibald et al., 2001; Clarren et al., 1978; Danis et al., 1981). Experimental models of FASD have confirmed that either late gestation (third trimester equivalent) binge (Bonthius and West, 1990; Bonthius and West, 1991; Goodlett et al., 1989; Phillips and Cragg, 1982; Pierce et al., 1989; West, 1993) or early- to mid-gestation chronic ethanol exposure impairs neuronal survival, growth, migration, and intracellular adhesion (Liesi, 1997; Maier and West, 2001; Miller, 1992, 1999; Minana et al., 2000; Olney et al., 2000; Soscia et al., 2006; Swanson et al., 1995; Yanni and Lindsley, 2000).

Successful neuronal migration requires integrity of growth factor—stimulated signaling through Erk MAPK, PI3 kinase—Akt, and cyclin-dependent kinase 5 pathways (Cantley, 2002; Chae et al., 1997; Johnson and Lapadat, 2002; Nikolic et al., 1998; Ohshima et al., 1999; Roymans and Slegers, 2001), adequate expression and function of intercellular adhesion molecules needed for growth cone guidance (Beggs et al., 1997), cross-talk signaling with extracellular matrix molecules (Porcionatto, 2006), activation of proteases used to degrade extracellular matrix (Romanic and Madri, 1994), and proper functioning of Wnt and Notch signaling mechanisms (Christiansen et al., 2000). Ethanol-impaired neuronal migration is likely due to multiple factors, including defects in intracellular signaling mechanisms that mediate neuronal—glial interactions (Guerri, 1998), neuronal adhesion (Luo and Miller, 1999; Minana et al., 2000), and glial cell functions required for myelination (Ozer et al., 2000).

Our research is focused on the inhibitory effects of ethanol on insulin and insulin-like growth factor (IGF) signaling because (1) insulin, IGF-1, and IGF-II receptors are abundantly expressed in the developing CNS; (2) signaling through these receptors mediates diverse neuronal functions, including survival and motility (de la Monte and Wands, 2005); and (3) neurotoxic effects of ethanol target insulin- and IGF-stimulated Erk MAPK and PI3 kinase—Akt signaling (de la Monte and Wands, 2005; de la Monte et al., 2001; Hallak et al., 2001; Soscia et al., 2006; Zhang et al., 1998). Previous studies linked ethanol inhibition of insulin/IGF signaling to reduced neuronal survival (de la Monte and Wands, 2002; de la Monte et al., 2001; Zhang et al., 1998) due to increased apoptosis (de la Monte et al., 2001; Ikonomidou et al., 2000; Zhang et al., 1998) and/or mitochondrial dysfunction (de la Monte and Wands, 2002; de la Monte et al., 2001; Ramachandran et al., 2001). Because previous studies demonstrated that aspartyl (asparaginyl)- β -hydroxylase (AAH), an important mediator of cell motility (Maeda et al., 2003; de la Monte et al., 2006; Sepe et al., 2002), is regulated by insulin and IGF signaling (Cantarini et al., 2006; Lahousse et al., 2006; de la Monte et al., 2006), and abundantly expressed in immature neuronal cells (Lahousse et al., 2006; Sepe et al., 2002), we hypothesized that ethanol-impaired neuronal migration was mediated in part by inhibition of insulin/IGF-stimulated AAH expression.

AAH is an ~86 kD type 2 transmembrane protein and member of the α -ketoglutarate—dependent dioxygenase family that includes prolyl-3, prolyl-4, and lysyl hydroxylases (Jia et al., 1992; Lavaissiere et al., 1996; Wang et al., 1991). AAH catalyzes post-translational hydroxylation of β carbons of specific aspartate and asparagine residues in epidermal growth factor—like domains present in proteins, such as Notch and Jagged, which have known roles

in cell growth, differentiation, and neuronal migration during development, and in extracellular matrix molecules, such as tenascin (Lavaissiere et al., 1996), which mediate adhesion, motility, and cell process extension (Goldbrunner et al., 1996; Merzak et al., 1995; Tucker and Chiquet-Ehrismann, 2006). AAH's carboxyl region can be proteolytically cleaved to generate ~52 or ~56 kD catalytically active fragments (Jia et al., 1992, 1994; Wang et al., 1991). Site-directed mutagenesis studies demonstrated that the ⁶⁷⁵His residue present in the C-terminal fragment is essential for catalytic activity (Dinchuk et al., 2000; Jia et al., 1992).

AAH's role as a mediator of cell motility was suggested by the findings that molecular silencing of the AAH gene with small interfering (si) RNA inhibited cell motility, whereas overexpression of AAH increased cell motility (Cantarini et al., 2006; de la Monte et al., 2006). The AAH gene is regulated by insulin and IGF signaling through insulin receptor substrate (IRS)—dependent pathways that activate Erk MAPK and PI3 kinase—Akt (Cantarini et al., 2006; de la Monte et al., 2006; Lahousse et al., 2006). However, AAH may also be regulated by post-translational mechanisms, because chemical inhibition of GSK-3 β by LiCl increases AAH protein without altering its mRNA levels (Lahousse et al., 2006). Because insulin and IGF signaling pathways are major targets of ethanol neurotoxicity (de la Monte and Wands, 2002; de la Monte et al., 2001; Xu et al., 2003; Zhang et al., 1998), we hypothesized that in FASD, ethanol-impaired neuronal migration was linked to inhibition of insulin/IGF-stimulated AAH expression and/or function. Herein, we used in vitro primary rat cerebellar granule neuron cultures and in vivo animal models of chronic gestational exposure to ethanol to characterize the mechanisms of ethanol-impaired neuronal migration in relation to AAH expression and function.

Experimental procedures

In vivo model of chronic ethanol exposure

Pregnant Long—Evans rats were pair-fed isocaloric liquid diets (BioServ, Frenchtown, N.J.) containing 0, 8, 18, 26, or 37% ethanol by caloric content (Chu et al., 2007; Soscia et al., 2006). The liquid diets began on gestation day 6 and continued until delivery of all pups. This approach was used because earlier periods of in utero ethanol exposure leads to excessive fetal loss due to impaired placentation (Gundogan et al., 2008). Rats were monitored daily to ensure equivalent caloric consumption and maintenance of body weight. Chow-fed controls were simultaneously studied. Maternal blood ethanol concentrations (mM) measured on the day of delivery were (mean $\pm$ standard deviation): 0, 34.9 $\pm$ 11.6, 93.6 $\pm$ 25.1, 140.2 $\pm$ 37.2, and 228.7 $\pm$ 49.7 in rats fed with the 0, 8, 18, 26, or 37% ethanol-containing diets, respectively, as previously reported (Soscia et al., 2006).

Cerebella were harvested on postnatal day 1 to examine the effects of ethanol on AAH expression. Cerebellar tissue was studied because it represents a major target of ethanol neurotoxicity (de la Monte et al., 2005; Lewis, 1985; Mattson et al., 2001; Volk, 1984). Fresh tissue was snap frozen in a dry ice-methanol bath and then stored at -80°C for mRNA and protein studies. In addition, cerebella were fixed, embedded in paraffin, and processed for histopathology and immunohistochemical staining (Soscia et al., 2006). The Lifespan-Rhode Island Hospital IACUC committee approved these procedures and the use of rats in experiments.

Protein studies

AAH immunoreactivity was detected in rat brains and primary cerebellar neuron culture homogenates with the A85G5 and A85E6 monoclonal antibodies (mAbs) (Supplementary Fig. 1). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) immunoreactivity was examined because it is an insulin-responsive gene (Alexander et al., 1992) that is inhibited by ethanol

(de la Monte and Wands, 2002). β -Actin and p85 subunit of PI3 kinase served as negative controls. Immunoreactivity was measured in cellular homogenates by Western blot analysis and enzyme-linked immunosorbant assays (ELISA; Cohen et al., 2007; Soscia et al., 2006; Xu et al., 2003). Protein concentrations were measured with the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The only modification of the original direct ELISA protocol was that immunoreactivity was detected with Amplex Red fluorophore (Ex 530/Em 590) (Pierce, Rockford, IL) and measured in a Spectra-max M5 microplate reader (Molecular Dynamics, Inc., Sunnyvale, CA). Antibody-binding specificity was assessed by substituting negative control, nonrelevant mAb to human Hepatitis B Surface antigen for the AAH mAb, omitting either the primary or secondary antibody, or coating the wells with 3% BSA in Tris-buffered saline (TBS) instead of sample.

Histologic sections of cerebella were stained with hematoxylin and eosin. Adjacent sections were immunostained with the A85G6 and A85E6 mAbs to AAH (Supplementary Fig. 1) as previously described (Soscia et al., 2006). Briefly, after de-paraffinization and re-hydration through graded ethanol solutions, the histologic sections were treated with 3% H_2O_2 in 60% methanol for 30 min at room temperature to quench endogenous peroxidase activity. Nonspecific binding sites were blocked by a 1 h, room temperature incubation with 2% normal mouse serum in 0.5% BSA and 0.1% Tween-20. The slides were incubated overnight at 4°C with 0.5 μ g/mL of primary antibody diluted in TBS containing 0.05% Tween-2 and 0.5% BSA (TBST—BSA). Immunoreactivity was detected using horse-radish peroxidase (HRP) polymer-conjugated secondary antibody (Dako, Carpinteria, CA) and diaminobenzidine as the chromogen (Vector Laboratories, Burlingame, CA) (Soscia et al., 2006). All histologic sections were immunostained simultaneously using the same batch preparations of primary and secondary antibodies and chromogen and under identical conditions. The sections were counter-stained with hematoxylin and examined by light microscopy under code to evaluate the presence and distribution of AAH immunoreactivity within the cerebellar cortex.

Quantitative (q) RT-PCR assays

Total RNA was isolated from cerebellar tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the AMV First Strand cDNA synthesis kit (Roche Diagnostics Corporation, Indianapolis, IN) and random oligodeoxynucleotide primers. Gene expression was measured by qRT-PCR analysis as previously described (Xu et al., 2003), except that amplified signals were detected and analyzed using the Mastercycler ep real-plex instrument and software (Eppendorf AG, Hamburg, Germany). Gene-specific primer sequences are listed in Supplementary Table 1.

Primary rat cerebellar neuron cultures

Primary neuronal cultures were generated with postnatal day 8 rat pup cerebella (de la Monte et al., 2005; Nikolic et al., 1996) and maintained with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 4 mM glutamine, 10 mM nonessential amino acid mixture (Gibco-BRL, Grand Island, NY), 25 mM KCl, and 50 mM glucose. For ethanol treatment, cultures were placed in sealed humidified chambers in which 50 mM ethanol was supplied to both the culture medium and reservoir tray (Adickes et al., 1988; Karl and Fisher, 1993). Control cultures were identically treated but with only water added to the reservoir tray. The chambers were flushed with gas containing 75% nitrogen, 20% oxygen, and 5% carbon dioxide, and the medium was changed daily with fresh additions of ethanol to both the medium and reservoir tray. Cultures were incubated in the chambers for up to 96 h at 37°C. To measure responsiveness to growth factor stimulation, the cells were serum-starved for 16 h (starting after 60 h in the chambers), and then stimulated with 10 nM insulin, 10 nM IGF-I, or vehicle for the last 24 h incubation in the chambers.

Cellular ELISA

A cellular ELISA was used to measure immunoreactivity directly in cultured cells (96-well plates) (de la Monte et al., 1999). The only modification of the original protocol was that immunoreactivity was detected with the Amplex Red fluorophore (Ex 530/Em 590; Pierce, Rockford, IL), and measured in a Spectramax M5 microplate reader (Molecular Dynamics, Inc., Sunnyvale, CA). Cell density was assessed by measuring fluorescence after staining the cells with Hoechst H33342 (Ex360 nm/Em460 nm; Molecular Probes, Eugene, OR). The calculated ratios of fluorescence immunoreactivity to H33342 were used for intergroup comparisons. At least eight replicate cultures were analyzed in each experiment.

Directional motility assay

Directional motility was measured using the ATP Luminescence—Based Motility—Invasion (ALMI) assay (de la Monte et al., 2002). Briefly, serum-free culture medium containing 10 nM insulin or 10 nM IGF-I was placed in the bottoms of blind well chambers (Neuro Probe, Gaithersburg, MD), and 8- μ m pore diameter polycarbonate filters divided the upper and lower chambers. Hundred thousand viable freshly isolated cerebellar granule neurons from P1 rat pups were seeded into the upper chambers and cell migration was allowed to proceed for 4 h at 37°C in a CO₂ incubator. Cells collected from the upper chambers (nonmotile), under surfaces of the filters (motile adherent), and bottoms of the wells (motile nonadherent) were quantified using ATPLite reagent (Perkin—Elmer, Waltham, MA) (de la Monte et al., 2002). Luminescence was measured in a TopCount Machine (Perkin—Elmer, Waltham, MA). The percentages of nonmotile, motile adherent, motile nonadherent cells in eight replicate assays were calculated and used for statistical analysis. Because this assay separately quantifies motile-adherent and motile-nonadherent subpopulations, it provides information about both motility and cell adhesion. For example, treatments that enhance cell motility without improving cell adhesion could lead to increased percentages of motile nonadherent cells and virtually no change in the percentages of motile-adherent cells. Because ethanol inhibits both cell motility and cell adhesion, it was of interest to determine if measures to increase AAH protein expression would result in increased percentages of motile nonadherent, motile adherent, or both populations of cells.

Analysis of AAH phosphorylation

Subsequence analysis of the translated AAH protein identified 24 potential phosphorylation sites, 13 of which have the consensus sequence for GSK-3 β (Supplementary Fig. 2). To determine if AAH protein can be phosphorylated, in vitro kinase assays were performed in 50 μ L reactions containing recombinant AAH protein (150 ng), recombinant catalytically active GSK-3 β (1 μ g) or MEK (1 U; negative control), [γ -³²P]ATP (1 μ Ci), and Mg/ATP cocktail (100 mM ATP, 75 mM MgCl₂) in reaction buffer (final concentration: 20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT). Reactions were incubated for 20 min at 30°C with agitation, and stopped by adding 10 μ L 0.5 mM EDTA. After adding protein sample buffer (Ausubel et al., 2002), the reaction products were fractionated by SDS-PAGE and detected with a phosphorimager followed by film autoradiography. In vitro results were validated by examining AAH phosphorylation in metabolically labeled PNET2 human CNS neuronal and FOCUS hepatocellular carcinoma cells using previously generated FB50 and 15C7 mAbs to AAH (Lahousse et al., 2006; Lavaissiere et al., 1996). Cell lines were used in these studies because they express high levels of AAH, which facilitated the overall analysis. The FB50 and 15C7 mAbs were used because they are highly immunoreactive with human AAH proteins.

Source of reagents

Human insulin and recombinant IGF-I were obtained from Sigma—Aldrich (St. Louis, MO). QuantiTect SYBR Green PCR Mix was obtained from (Qiagen Inc, Valencia, CA). H33342 was purchased from Molecular Probes (Eugene, OR). ATPLite reagents were purchased from Perkin—Elmer (Boston, MA). All other fine chemicals and antibodies were purchased from either CalBiochem (Carlsbad, CA) or Sigma—Aldrich (St. Louis, MO).

Statistical analysis

Data depicted in the graphs represent the means $\pm$ standard error of the means for each group. Intergroup comparisons were made using Student's *t*-tests or analysis of variance (ANOVA) with the Tukey—Kramer post hoc test for significance. Statistical analyses were performed using the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysville, UT) and significant *P* values ($<.05$) are indicated over the graphs.

Results

Reduced AAH immunoreactivity in rat pup cerebella following chronic gestational exposure to ethanol

Histologic sections were immunostained with the A85G6 or A85E6 mAbs because A85G6 recognizes AAH, whereas A85E6 recognizes both AAH and Humbug. This approach was taken because AAH mediates motility (Cantarini et al., 2006; de la Monte et al., 2006; Lahousse et al., 2006; Sepe et al., 2002), whereas Humbug does not and instead appears to mediate cell adhesion (Cantarini et al., 2006; Lahousse et al., 2006), both of which are impaired in FASD (Goodlett et al., 2005; Minana et al., 2000; Ozer et al., 2000). The sections were examined under code and ranked according to the relative intensity of immunoreactivity. Subsequently, slides with similar levels of immunostaining reactions were placed into one of five groups, because eight cases each from the different ethanol exposure diets (0, 8, 18, 26, and 37%) were studied. After decoding the sections, it was evident that control cerebella (0% ethanol diet) had conspicuously higher levels of A85G6 and A85E6 immunoreactivity compared to ethanol-exposed cerebella. Chronic gestational exposure to ethanol yielded intensities of A85G6 and A85E6 immunoreactivity that were inversely graded relative to ethanol dose such that the lowest levels were observed in the 37% ethanol diet group, and the highest, but still reduced relative to control, were in the 8% ethanol diet group (Fig. 1). A85G6 and A85E6 immunoreactivities were most prominently localized in the granule cell layers of the cerebella. The A85G6 and A85E6 mAbs produced overlapping but somewhat dissimilar results. For example, the A85E6 mAb was associated with less intense cortical, and very low levels of white matter cell labeling compared to the A85G6 mAb. In addition, the A85G6 antibody produced a sharp delineation between the external granule cell layer and deeper cortical layers, whereas with the A85E6 mAb, the laminar demarcation was less pronounced. Finally, although A85G6 immunoreactivity was easily detected in cerebella from the 37% ethanol diet group, A85E6 immunoreactivity was virtually undetectable in adjacent sections (Fig. 1).

Quantification of ethanol-impaired AAH protein expression

The A85G6 mAb detected ~86 kD and ~110 kD bands corresponding with the expected sizes of full-length and probably post-translational modified AAH protein (Fig. 2A). Corresponding with the immunohistochemical staining results, the levels of A85G6 immunoreactivity were reduced in cerebella of ethanol-exposed pups, particularly in those from 24% and 37% ethanol diet groups (Fig. 2A). In contrast, p85 immunoreactivity (subunit of PI3 kinase) was not modulated by ethanol exposure (Fig. 2A). Digital image quantification of the Western blot signals demonstrated an inverse relationship between ethanol dose and A85G6 immunoreactivity (Fig. 2B). Inter-group comparisons of the mean levels of A85G6

immunoreactivity were statistically significant for the 18% and higher ethanol dose groups ($F = 21.4$; $P < .0001$; Fig. 2B). These findings were confirmed by direct ELISA measurement of A85G6 immunoreactivity ($F = 27.53$; $P < .0001$) (Fig. 2C). Similarly, Western blot analysis (Fig. 2D) and ELISA (data not shown) studies demonstrated significantly reduced levels of A85E6 immunoreactivity in ethanol-exposed relative to control cerebella, but unlike A85G6, the effects of ethanol were similar across all doses used ($F = 22.0$; $P < .0001$).

Ethanol-mediated reductions in AAH protein were not due to impairments in AAH mRNA expression

The qRT-PCR studies demonstrated that the chronic gestational exposure to ethanol did not significantly inhibit AAH mRNA expression (Fig. 2E). In fact, the mean levels of AAH mRNA were similar to controls, except in the 37% ethanol-diet group in which the levels were significantly increased ($F = 2.79$; $P = .049$) (Fig. 2E). The mean levels of 18S rRNA, which served as a loading control, were similar among the groups (Fig. 2F).

Chronic gestational exposure to ethanol impairs cerebellar granule neuron motility

Directional motility was measured in pooled samples of cerebellar granule neurons isolated from four litters each of P1 rat pups born to dams that had been fed with 0% (control) or 37% ethanol-containing diets. Either IGF-1 (10 nM) or insulin (10 nM) was supplied as the trophic factor in parallel studies, and directional motility was allowed to proceed for 4 h at 37°C. Note that for cell lines, directional motility assays are generally conducted over a period of 30 min, but with nontransformed cells, additional time is required to measure motility. Chronic gestational exposure to ethanol significantly reduced both IGF-I— and insulin—stimulated directional motility (Fig. 3A, B). This effect was mainly due to significant reductions in the percentages of migrated adherent cells, suggesting that ethanol inhibits both neuronal motility and adhesion. Because the assays were conducted in the absence of a matrix coating, the measurements reflect net directional motility rather than invasion.

In vitro experiments demonstrating ethanol inhibition of AAH protein expression

Primary cerebellar granule neuron cultures were used to examine the effects of ethanol (50 mM) on AAH expression. Western blotting with the A85G6 antibody detected the expected ~110 and ~86 kD bands corresponding to full-length and probably post-translational modified AAH protein (Fig. 4A). In contrast, the A85E6 antibody detected mainly a ~52–54 kD band (Fig. 4B) that could correspond to either cleaved AAH as previously reported (Korioth et al., 1994; Lavaissiere et al., 1996; Wang et al., 1991) or Humbug (Cantarini et al., 2006). Ethanol significantly reduced the levels of A85G6 and A85E6 immunoreactivity (Fig. 4C, D). Further studies demonstrated that ethanol also reduced GAPDH (Fig. 4C; positive control), but had no significant effect on β -actin (Fig. 4D; negative control) expression. Cellular ELISA results confirmed the findings obtained by Western blot analysis (Fig. 4E, F), including ethanol inhibition of GAPDH (Fig. 4G), and preservation of β -actin (Fig. 4H) expression in ethanol-treated cells.

Post-translational regulation of AAH

Because ethanol inhibition of AAH protein was not associated with reduced levels of its mRNA, we explored potential post-translational mechanisms of AAH regulation. We focused our investigations on the role of GSK-3 β —mediated phosphorylation of AAH because (1) multiple potential Serine phosphorylation sites with the consensus sequence for GSK-3 β were identified by subsequence analysis of the AAH protein (Supplementary Fig. 2); (2) LiCl inhibition of GSK-3 β was demonstrated to increase AAH protein without altering its mRNA (Lahousse et al., 2006); and (3) ethanol treatment increases GSK-3 β activity in neuronal cells (Xu et al., 2003). Metabolic labeling studies demonstrated that [32 P]-labeled proteins could be

immunoprecipitated from PNET2 and FOCUS hepatocellular carcinoma cell lysates using the FB50 + 15C7 mAbs to AAH (Lahousse et al., 2006; Lavaissiere et al., 1996), but not with nonrelevant SF25 mAb (Palumbo et al., 2002; Wilson et al., 1988) (Fig. 5A).

In vitro kinase assays constructed with recombinant human AAH protein, recombinant constitutively active kinases, and [γ - 32 P]ATP demonstrated that AAH could be phosphorylated directly by GSK-3 β , but not Erk MAPK (Fig. 5B). Western blot analysis demonstrated markedly reduced levels of phospho-Serine immunoreactivity in FB50 + 15C7 (AAH) immunoprecipitants of LiCl-treated (2 h) compared to vehicle-treated PNET2 cells (Fig. 5C), although levels of AAH protein were not modulated by the short-term treatment (Fig. 5C). Finally, to demonstrate specificity of the GSK inhibition on AAH phosphorylation, PNET2 cells were treated for 2 h with vehicle, 10 nM PD98059 (Erk mitogen-activated protein kinase [MAPK] inhibitor), 5 nM SB202190 (p38 MAPK inhibitor), 20 ng/mL Ly294002 (PI3 Kinase inhibitor), or 20 mM LiCl (GSK-3 β inhibitor), and AAH immunoreactivity was examined by Western blot analysis of phospho-enriched protein fractions that were obtained using the BDTM Phosphoprotein Enrichment kit (BD Biosciences, San Jose, CA). Those studies demonstrated similar levels of phospho-AAH in cells treated with vehicle, PD98059 or SB202190, increased phospho-AAH in cells treated with Ly294002, and reduced phospho-AAH in cells treated with LiCl (Fig. 5D). Note that Ly294002 inhibition of PI3 kinase typically results in increased GSK-3 β activity.

Partial rescue of ethanol-impaired AAH expression

To determine the effects of ethanol on AAH phosphorylation in primary cerebellar neuron cultures, phospho-Serine immunoprecipitates and total cell homogenates were subjected to Western blot analysis using the A85G6 mAb. Those studies demonstrated disproportionately higher relative levels of Serine phosphorylated compared to total AAH in ethanol exposed (50 mM $\times$ 96 h) relative to control cells (Fig. 6A). In contrast, p85 protein levels were similar for the two groups, indicating equal loading of protein samples (Fig. 6A).

Experiments were conducted to determine the degree to which ethanol-impaired AAH protein expression could be rescued by inhibiting GSK-3 β . In addition, because phosphorylation can target proteins for cleavage and degradation, and Caspase activity and apoptosis are increased in ethanol-exposed neuronal cells and brains (de la Monte and Wands, 2001; de la Monte et al., 2000, 2001; Heaton et al., 2003; Light et al., 2002; Luo et al., 1997; Miller, 1996; Oberdoerster et al., 1998; Ramachandran et al., 2001; Siler-Marsiglio et al., 2005; Xu et al., 2003), we sought to determine if AAH protein expression in ethanol-treated cerebellar neuron cultures could be restored to control levels by inhibiting Caspase activity. Aspartyl (asparaginy)l- β -hydroxylase, GAPDH, and β -actin were examined by Western blot analysis (Fig. 6B) and quantified by cellular ELISA (Fig. 6C—F). Inter group differences were assessed using analysis of variance (ANOVA) and Tukey post hoc tests of significance. Overnight treatment with 20 mM LiCl and/or 3 h treatment with 1 μ M z-VAD-fmk increased A85G6 ($F = 19.92$; $P = .0002$) and A85E6 ($F = 23.33$; $P < .0001$) immunoreactivity in ethanol-exposed cells to levels that were comparable to control (Fig. 6B—D). Also note that the combined treatments LiCl+z-VAD-fmk significantly increased A85G6 and A85E6 immunoreactivity in both control and ethanol-exposed cells (Fig. 6C, D). In contrast, GAPDH immunoreactivity was significantly reduced in all ethanol-exposed relative to control cultures ($F = 18.89$; $P = .0008$), and the adverse effects of ethanol were not significantly altered by LiCl, z-VAD-fmk, or both treatments (Fig. 6E). β -Actin levels were not significantly reduced by ethanol exposure, nor altered by LiCl, z-VAD-fmk, or both treatments (Fig. 6F).

Discussion

AAH expression is reduced in experimental FASD: correlation with impaired neuronal migration

Our interest in the effects of ethanol on AAH expression and CNS neuronal migration in FASD stems from the findings that: (1) AAH has a demonstrated role in regulating cell motility (Cantarini et al., 2006; Lahousse et al., 2006; Maeda et al., 2003; de la Monte et al., 2006; Sepe et al., 2002); (2) AAH and motility are regulated by insulin and IGF stimulation (Lahousse et al., 2006; de la Monte et al., 2006; Puglianiello et al., 2000); and (3) ethanol inhibits insulin- and IGF-mediated signaling in immature neuronal cells (de la Monte and Wands, 2002; de la Monte et al., 2005; Dees et al., 2005; Hallak et al., 2001; Seiler et al., 2001; Tatenio et al., 2004; Xu et al., 2003; Xu et al., 1995). Our in vivo rat model of FASD has a striking phenotype with respect to ethanol dose-dependent abnormalities in cerebellar structure, including hypoplasia and impaired neuronal migration (Soscia et al., 2006). Correspondingly, we observed ethanol dose-dependent reductions in cerebellar AAH immunoreactivity, and significant impairments in insulin- and IGF-I-stimulated directional motility in granule neurons isolated from ethanol-exposed rat pup cerebella. In addition to reduced motility, we observed that chronic in vivo ethanol exposure mainly reduced the percentages of migrant-adherent cells, consistent with previous reports indicating that ethanol impairs neuronal cell adhesion mechanisms (Goodlett et al., 2005; Minana et al., 2000; Seiler et al., 2001). Therefore, ethanol inhibition of insulin- and IGF-I-stimulated CNS neuronal migration in FASD is associated with reduced levels of AAH protein expression. Additional studies are required to evaluate the role of ethanol-inhibition of Humbug in relation to the associated impairments in neuronal adhesion.

The mechanism by which AAH mediates cell motility is not completely understood. Previous studies demonstrated that AAH signals through Notch and Jagged (Cantarini et al., 2006; Dinchuk et al., 2002), which mediate a broad array of functions during development (Cornell and Eisen, 2005), including cell migration (Bolos et al., 2007). Notch signaling promotes neuronal migration during development through its regulation of radial glial progenitor cell function (Ever and Gaiano, 2005; Kiyota et al., 2008) and expression of neuronal cell adhesion molecule (N-CAM) (Lieber et al., 1992). Therefore, AAH hydroxylation of Notch and Jagged could mediate neuronal migration and adhesion during development by enhancing function of radial glia and expression of N-CAM.

The amino terminus of AAH is highly homologous with its related molecules, Humbug and Junctin, which lack AAH's catalytic domain, but function by releasing intracellular stores of calcium from the endoplasmic reticulum or sarcoplasmic reticulum (Dinchuk et al., 2000; Treves et al., 2000; Wetzel et al., 2000). Therefore, the N-terminus of AAH may also function by increasing levels of intracellular calcium. In this regard, previous studies demonstrated that ethanol-impaired neuronal migration is caused in part by disruption of second messenger pathways, including calcium signaling/homeostasis (Kumada et al., 2007; Liesi, 1997; Webb et al., 2003). Transforming growth factor β 1 (TGF- β 1) stimulates cell motility by increasing cytoplasmic calcium release from intracellular stores (Chow et al., 2008), and ethanol disrupts TGF- β 1 signaling mechanisms. Therefore, the inhibitory effects of ethanol on AAH could lead to reduced neuronal motility by: (1) inhibiting downstream signaling through Notch and (2) dysregulating intracellular calcium release mechanisms. This hypothesis is currently under investigation in our laboratory.

Role of ethanol-impaired insulin/IGF signaling in relation to reduced AAH expression in cerebellar granule neurons

The effects of ethanol on AAH expression were also examined in primary cerebellar granule neuron cultures. Those studies showed that even after short-term ethanol exposure (96 h), insulin- and IGF-responsive gene expression, including AAH and GAPDH, was significantly impaired, corresponding with previous findings in other cell culture models (de la Monte and Wands, 2002; de la Monte et al., 2001, 2005; Hallak et al., 2001; Seiler et al., 2001; Xu et al., 1995, 2003). Because insulin/IGF and AAH have demonstrated roles in neuronal motility, ethanol inhibition of insulin/IGF signaling and AAH expression may represent critical mediators of impaired CNS neuronal migration in FASD.

Previous studies demonstrated that insulin and IGF signaling through PI3 kinase—Akt or Erk MAPK increase AAH mRNA and protein expression (Cantarini et al., 2006; Lahousse et al., 2006; de la Monte et al., 2006), whereas inhibition of GSK-3 β increases directional motility and AAH protein, but not AAH's mRNA (Lahousse et al., 2006). Although ethanol-mediated impairment of neuronal motility could be explained by its inhibitory effects on insulin/IGF signaling through PI3 kinase—Akt and/or Erk MAPK (Bhave et al., 1999; de la Monte and Wands, 2002; Luo and Miller, 1998; Xu et al., 2003; Zhang et al., 1998), the findings herein suggest that ethanol's inhibitory effects on AAH are mainly mediated at a post-transcriptional or post-translational level because AAH mRNA levels were similar to, or significantly increased relative to control. Correspondingly, we have shown that AAH protein can be phosphorylated by GSK-3 β , ethanol-mediated increases in AAH phosphorylation decrease AAH's protein expression, ethanol increases GSK-3 β activity, and chemical inhibition of GSK-3 β increases both AAH protein and motility in neuronal cells. Moreover, recent preliminary studies designed to characterize the mechanism of increased neuronal AAH mRNA after chronic high-level ethanol exposure revealed that the associated increase in oxidative stress results in activation of hypoxia-inducible factor-1 α (HIF-1 α), which in turn, is an upstream positive regulator of AAH mRNA (Lawton, M. and de la Monte, S.M., unpublished). Finally, there is growing evidence that hypoxia, oxidative stress, and even Caspase activation can modulate cell motility by altering the cyclical polymerization—depolymerization of cytoskeletal filaments (Cheng et al., 2008; Das et al., 2008; Li et al., 2007; Rojas et al., 2006).

GSK-3 β —mediated AAH protein phosphorylation

Given that ethanol increases GSK-3 β activity in neuronal cells (de la Monte and Wands, 2002; Sheu et al., 1998; Takadera and Ohyashiki, 2004; Xu et al., 2003), and AAH has consensus sequences for GSK-3 β —mediated Serine phosphorylation at multiple sites (Supplementary Fig. 2), we hypothesized that ethanol treatment would lead to increased GSK-3 β phosphorylation of AAH. Correspondingly, in vitro studies demonstrated that: (1) AAH protein is phosphorylated in neuronal cells; (2) AAH recombinant protein can be phosphorylated by GSK-3 β and (3) Serine phosphorylation of AAH is reduced by LiCl treatment. Together, these results suggest that AAH can be post-translationally modified by GSK-3 β —mediated phosphorylation, raising the possibility that AAH protein may be regulated by its phosphorylation state. Moreover, because ethanol increases GSK-3 β activity (Acquaah-Mensah et al., 2002; de la Monte and Wands, 2002; Takadera and Ohyashiki, 2004; Xu et al., 2003), it is conceivable that the ethanol-associated reductions in AAH protein could be mediated by increased GSK-3 β phosphorylation. The latter point is reinforced by the finding of higher levels of Serine phosphorylated AAH in ethanol-exposed compared with control cerebellar neurons.

GSK-3 β and caspases negatively regulate AAH in ethanol-exposed immature cerebellar neurons

One potential consequence of increased phosphorylation is increased degradation of the specific protein. Exploratory studies focused our investigations on the role of Caspases because ethanol increases Caspase activation in brain (Goodlett et al., 2005; Xu et al., 2003), and Caspase cleavage can be triggered by changes in protein phosphorylation state (Li et al., 2001; van de Water et al., 2000). Correspondingly, studies herein demonstrated partial rescue of ethanol-impaired AAH protein expression in cells treated with LiCl to inhibit GSK-3 β , or Z-VAD.fmk to inhibit global Caspases, and further increases in AAH protein in cells that were treated with both LiCl and Z-VAD.fmk. These results suggest that in ethanol-exposed neuronal cells, AAH phosphorylation by GSK-3 β renders AAH protein more susceptible to proteolytic degradation by Caspases. Therefore, in the context of FASD, impaired CNS neuronal migration could be mediated by constitutively reduced neuronal levels of AAH protein caused by inhibition of insulin/IGF signaling through PI3 kinase—Akt and attendant increased levels of GSK-3 β , combined with higher levels of Caspase activity afforded by increased oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Impaired cerebellar neuron migration in a graded model of fetal alcohol spectrum disorder is associated with reduced aspartyl (asparaginy)- β -hydroxylase (AAH) immunoreactivity. Pregnant Long—Evans dams were pair-fed with isocaloric liquid diets containing (A, F, K, P) 0%, (B, G, L, Q) 8%, (C, H, M, R) 18%, (D, I, N, S) 26%, or (E, J, O, T) 37% ethanol by caloric content from gestation day 6 through delivery. Cerebella from postnatal day 1 pups were fixed, embedded in paraffin (mid-sagittal plane), and immunostained with the A85G6 (A—J) or A85E6 (K—T) mAbs using the ABC method with DAB as the chromogen (brown precipitate). Sections were counterstained with hematoxylin. Representative low (A—E and K—O; original $\times 40$) and high (F—J and P—T; original $\times 200$) magnification images illustrate effects of ethanol dose on AAH immunoreactivity, A85G6 binds to the C-terminus of AAH, and A85E6 binds to the N-terminus of AAH protein. EGL, external granule cell layer; IGL, internal granule cell layer; WM, white matter. Bars indicate approximate widths of the EGL and IGL.

Fig. 2.

Effects of chronic gestational exposure to ethanol on aspartyl (asparaginyl)- β -hydroxylase (AAH) expression. Protein homogenates of cerebella from P1 rat pups exposed to 0, 8, 18, 26, or 37% ethanol via pregnant dams' liquid diets (see Experimental procedures) were used to measure AAH protein expression. (A) Example Western blot analysis using the A85G6 mAb. The blots were stripped and re-probed with polyclonal antibodies to the p85 subunit of PI3 kinase as a loading control. (B) Digital image quantification of A85G6 Western blot signals (~86 kD band-arrow), including three samples per ethanol dose. Levels of immunoreactivity were measured in arbitrary densitometry units (DU). (C) A85G6 immunoreactivity measured by enzyme-linked immunosorbent assay (eight samples/group) using HRP-conjugated

secondary antibody, Amplex Red fluorophore, and an M5 microplate reader (Ex 530 nm/Em 590 nm). (D) Western blot analysis was also performed using the A85E6 mAb (blot not shown). Graph depicts results of digital image quantified signals reflecting A85E6 immunoreactivity (~52 kD band), including three samples per ethanol dose. (E) AAH mRNA and (F) 18S rRNA levels were measured by qRT-PCR. AAH mRNA levels were normalized to 18S measured in corresponding samples. All graphs depict mean $\pm$ standard error of the mean of results. Statistical comparisons were made using one-way analysis of variance and the post hoc Tukey—Kramer test for significance. Significant *P* values relative to control (0% ethanol) are indicated over the bars. **P* < .05.

Fig. 3.

Chronic gestational exposure to ethanol impairs motility in cerebellar neurons. Freshly isolated cerebellar granule neurons from P1 pups born to dams exposed to a 0 or 37% ethanol containing diet were used to measure directional motility with the ATP luminescence—based motility and invasion assay (see Experimental procedures). Insulin-like growth factor-I (IGF-I) (10 nM) or insulin (10 nM) was supplied as the trophic factor. Cells were allowed to migrate through 8- μ m pore diameter polycarbonate filters for 4 h at 37°C. Then, cells remaining in the upper chamber (nonmotile), the under surface of the filter (migrated adherent), or bottoms of the wells (migrated nonadherent) were quantified using ATP-Lite reagent. The motility indexes, that is percentages of cells in each compartment, as well as total percentages of migrated cells (migrated adherent + migrated nonadherent) were calculated. Results (mean $\pm$ standard deviation) from 12 replicate assays are depicted graphically. Intergroup comparisons were made using the Student's *t*-test **P* < .05.

Fig. 4.

In vitro ethanol exposure inhibits neuronal aspartyl (asparaginy)l- β -hydroxylase expression. Cultured cerebellar granule neurons were exposed to 0 or 50 mM ethanol for 96 h in sealed humidified chambers. Proteins homogenates were used to measure (A) A85G6, (B) A85E6, (C) glyceraldehydes-3-phosphate dehydrogenate (GAPDH) and (D) β -actin immunoreactivity by Western blot analysis. Representative results are shown. Positions of molecular weight standards are indicated to the left of the blots. Levels of (E) A85G6, (F) A85E6, (G) GAPDH, and (H) β -actin immunoreactivity were also examined by cellular ELISA using horseradish peroxidase-conjugated secondary antibody and Amplex Red fluorophor ($N=12$ samples/group). Fluorescence light units (Ex 530 nm/Em 590 nm) were measured in an M5 microplate reader. Results were normalized to H33342 fluorescence to correct for differences in cell density. Intergroup statistical comparisons were made using the Student's t -test and significant P values are indicated over the bars.

Fig. 5.

Aspartyl (asparaginyl)-β-hydroxylase (AAH) protein phosphorylation studies. (A) Human FOCUS hepatocellular carcinoma (Lanes 1 and 3) and PNET2 CNS—derived neuronal cells (Lanes 2 and 4) were metabolically labeled with [32 P] orthophosphoric acid and FB50+15C7 (AAH; Lanes 1 and 2) or SF25 (nonrelevant; Lanes 3 and 4) mAb immunoprecipitants were fractionated by SDS-PAGE and detected by autoradiography. (B) In vitro phosphorylation of AAH was assessed by incubating recombinant full-length AAH protein and vehicle (Control), catalytically active GSK-3 β , or Erk MAPK with [γ - 32 P] ATP. Reactants were fractionated by SDS-PAGE and detected by phosphorimage analysis. (C) Cultured PNET2 cells were treated with vehicle (Con) or 20 mM LiCl (inhibitor of GSK-3 β) for 2 h. AAH protein was immunoprecipitated with the FB50+15C7 mAbs, and the immunoprecipitants were subjected to Western blot analysis with anti-phospho-Serine (pS-AAH). Blots were stripped and re-probed with rabbit polyclonal anti-AAH (AAH). Immunoreactivity was revealed with horseradish peroxidase-conjugated secondary antibodies, ECL reagents, and digital imaging. (D) PNET2 cells were treated with vehicle (Lane 1), 10 nM PD98059 (Lane 2), 5 nM SB202190 (Lane 3), 20 ng/mL Ly294002 (Lane 4), or 20 mM LiCl (Lane 5). Cellular proteins fractionated by phospho-enrichment were subjected to Western blot analysis with the A85G6 mAb to AAH. In each panel, positions of molecular weight standards are shown at the left.

Fig. 6.

Restoration of aspartyl (asparaginy)- β -hydroxylase (AAH) expression in ethanol-exposed cerebellar neurons by treatment with LiCl (GSK-3 β ; inhibitor) and/or Z-VAD-fmk (global Caspase inhibitor). (A) Phospho-Serine (PS) immunoprecipitants of protein homogenates (250 μ g) obtained from control or ethanol-exposed (50 mM $\times$ 96 h) rat cerebellar neuron cultures were subjected to Western blot (WB) analysis using the A85G6 mAb to AAH. In addition, the protein homogenates were evaluated by direct Western blot analysis (20 μ g protein/lane) to measure total AAH protein (A85G6) and p85 subunit of PI3 kinase (loading control). AAH (A85G6, A85E6), glyceraldehydes-3-phosphate dehydrogenate (GAPDH), and β -actin immunoreactivities were examined by (B) Western blot analysis and (C–F) cellular enzyme-linked immunosorbent assay (ELISA) in control and ethanol-treated cerebellar neuron cultures that were treated with vehicle, 20 mM LiCl (to inhibit GSK-3 β), 1 μ M Z-VAD-fmk (to inhibit global Caspases), or LiCl+Z-VAD-fmk. Cells were treated with LiCl overnight and with Z-VAD-fmk for 3 h. For cellular ELISAs, immunoreactivity was normalized to H33342 fluorescence, which reflects cell density. Fluorescence light units (FLU) were measured in an M5 microplate reader. Eight replicate samples were used per group. Intergroup statistical comparisons were made using analysis of variance with post hoc Tukey–Kramer significance tests. $*P < .05$ and $**P < .001$ relative to corresponding paired control. P values indicated over horizontal bars reflect significant differences relative to corresponding vehicle-treated cultures.