ELSEVIER

Developmental Brain Research 138 (2002) 177–185

DEVELOPMENTAL BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Effects of early postnatal ethanol intubation on GABAergic synaptic proteins

Shu-Huei Hsiao[a], Alan R. Parrish[a], Sang-Soep Nahm[b], Louise C. Abbott[b], Brian A. McCool[a], Gerald D. Frye[a,*]

[a]Department of Medical Pharmacology and Toxicology, College of Medicine, Texas A&M University System Health Science Center, College Station, TX 77843-1114, USA

[b]Department of Veterinary Anatomy, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA

Accepted 30 July 2002

Abstract

Fetal alcohol syndrome includes brain damage from aberrant synaptogenesis, altered cell–cell signaling and blunted plasticity in surviving neurons. Distortion of neurotrophic GABA signals by ethanol-mediated allosteric modulation of $GABA_A$ receptor ($GABA_AR$) activity during brain maturation may play a role. In this regard, early postnatal binge-like ethanol treatment on postnatal days (PDs) 4–9 acutely inhibits whole cell $GABA_AR$ Cl^- current and subsequently blunts $GABA_AR$ function in medial septum/diagonal band (MS/DB) neurons and cerebellar Purkinje cells [*Dev. Brain Res.* 130 (2001) 25–40; *Brain Res.* 810 (1998) 100–113; *Brain Res.* 832 (1999) 124–135]. In light of these functional changes, we hypothesized that ethanol treatment also would decrease levels of proteins important for assembly of GABAergic synapses in maturing brain. To test this relationship, binge-like ethanol intubation was administered to rat pups on PDs 4–9 producing peak blood ethanol concentrations in the range of 302.5 ± 6.3 mg/dl. GABAergic synaptic proteins were measured in brain tissue on PDs 13–14 when $GABA_AR$ currents in individual MS/DB neurons are reduced, but those of cerebellar Purkinje neurons are not yet altered [*Dev. Brain Res.* 130 (2001) 25–40; *Brain Res.* 810 (1998) 100–113; *Brain Res.* 832 (1999) 124–135]. Surprisingly, ethanol did not decrease protein levels of $GABA_AR$ $\alpha1/\beta2$ subunits, GAD_{67} or gephyrin in MS/DB at this time when whole cell recordings indicate $GABA_AR$ function is impaired in acutely dissociated individual neurons. However, in cerebellum where ethanol treated Purkinje cell $GABA_AR$ function remains normal on PDs 13–14 [*Brain Res.* 832 (1999) 124–135], reduced levels of several GABAergic synaptic proteins including: GAD_{67}, $GABA_AR$ $\alpha1$ subunit, ClC-2 a voltage-gated Cl^- channel, synaptotagmin a synaptic vesicle protein, and N-cadherin, a synapse associated cell adhesion molecule, were found. These results indicate that binge-like ethanol exposure differentially decreases GABAergic synaptic proteins in some brain areas in a pattern that does not parallel reductions in $GABA_AR$ function of individual neurons that survive this ethanol insult.
© 2002 Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behaviour

Topic: Drugs of abuse: alcohol, barbiturates, and benzodiazepines

Keywords: Fetal alcohol syndrome; Western blot; Adhesion molecule; $GABA_A$ receptor subunit; Development

1. Introduction

The fetal alcohol syndrome (FAS) results from ethanol consumption during pregnancy and is a leading cause of birth defects including brain damage and dysfunction known as 'alcohol-related neurodevelopmental disorder' (ARND) [51]. Many indices of brain injury including cognitive impairment and motor incoordination observed in humans are convincingly modeled in animals [7,16,61]. This work suggests that neurons surviving the initial ethanol insult may exhibit lasting impairment in the form of aberrant connectivity, altered cell–cell signaling and blunted plasticity [7,24,61]. The molecular mechanism(s) by which ethanol distorts neuronal development are not well understood, but one target is the $GABA_A$ receptor ($GABA_AR$). $GABA_AR$s mediate many inhibitory actions of

*Corresponding author. Tel.: +1-979-845-2888; fax: +1-979-845-0699.

E-mail address: gdfrye@tamu.edu (G.D. Frye).

0165-3806/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0165-3806(02)00470-4

GABA in adult brain and are positively modulated by many central nervous system (CNS) depressants including ethanol [5,54]. In immature brain, activation of $GABA_ARs$ depolarizes cells and can increase intracellular Ca^{2+} through voltage-sensitive channels, to influence neuronal differentiation, migration, neurite formation and synaptogenesis [4,10,33]. GABA acting primarily through $GABA_ARs$ increases neurite growth and synaptogenesis as well as the expression of $GABA_ARs$ in cultured cerebellar granule cells [9]. Synaptogenesis in the CNS is paralleled by a transition from $GABA_AR$ excitation to inhibition as differential expression of Cl^- channels and transporters causes a hyperpolarizing shift of the Cl^- reversal potential [13,20,50,57]. $GABA_AR$ subunit expression also shifts as receptors increasingly becoming synaptic transducers [19,37]. Ethanol modulation of excitatory $GABA_ARs$ during development could inhibit trophic influences of GABA and disrupt neuronal circuit formation. Consistent with this view are several recent reports which indicate that perinatal ethanol exposure can modify $GABA_AR$ level and function in developing brain with long-lasting consequences [1–3,26–28,31].

In this regard, ethanol has been shown to directly inhibit early postnatal $GABA_ARs$ on medial septum/diagonal band (MS/DB) and cerebellar Purkinje neurons [27,28], an action which could blunt GABAergic trophic actions. Repeated binge-like ethanol exposure of rat pups modeling human third trimester FAS [61] delays subsequent up-regulation of $GABA_AR$ chloride currents in these neurons [26–28]. The present study tested the hypothesis that functional reductions in $GABA_AR$ currents measured in individual CNS neurons would predict a decrease in proteins involved in GABAergic synapse formation after postnatal ethanol exposure. To test this idea, levels of the α1 and β2 $GABA_AR$ subunit were measured, since these peptides along with the γ2 subunit are thought to form a majority of $GABA_ARs$ across the maturing CNS including those on MS/DB and Purkinje neurons [19,37,41,63]. Gephyrin, a tubulin binding protein, also was studied since it participates in anchoring postsynaptic strychnine-sensitive glycine and $GABA_ARs$ [17,36], while ClC-2, a voltage-gated Cl^- channel, was included because it is highly expressed in MS/DB and Purkinje neurons and plays a role in regulating Cl^- gradients involved in $GABA_AR$ excitatory responses [55]. Finally, synaptotagmin, the synaptic vesicle SNARE complex protein [18], N-cadherin, a transmembrane cell adhesion molecule present during synapse formation [11,29] and α-/β-catenins which bind intracellularly to N-cadherin [11,60,64], were examined as general markers of synaptic development. The results indicate that postnatal day (PD) 4–9 binge-like ethanol exposure differentially decreases GABAergic synaptic proteins across brain regions on PDs 13–14. These changes likely reflect significant cell damage and neuronal loss in a particular brain area rather than impaired $GABA_AR$ function in individual neurons that survive the ethanol insult.

2. Materials and methods

2.1. Experimental animals and postnatal alcohol treatment

Timed-pregnant Sprague–Dawley rats (Harlan) were maintained under standard conditions (22–25 °C; 12/12 h light/dark cycle; ad lib standard rat chow and water). All care and handling was in accord with policies of Texas A&M University Laboratory Animal Care Committee. Early postnatal binge-like ethanol treatment was administered to rat pups as previously reported [26]. Two days after birth (PD 2) litters were culled to eight pups. Over PDs 4–9 one half of the pups (ethanol) were administered ethanol-containing milk [62] in two doses 2 h apart via manual oral-gastric intubation (total dose=4.9 g/kg/day at 11.0%, v/v, ethanol). This group also received a third feeding of milk 'alone' 2 h later. Blood ethanol concentrations (BECs) were measured on PD 6, 90 min after the second ethanol dose. The other half of the pups (control) were intubated three times without milk and had tail blood samples taken on the same schedule as ethanol animals to control for stress.

2.2. Protein measurements

Western blot analysis was carried out using a standard protocol as previously reported [23] with minor modifications. In general, each experiment compared pooled brain tissue from a litter of four ethanol and four control pups and was independently replicated 4–9 times. Brain regions including MS/DB, cerebellum, cerebral cortex, hippocampus and brain stem were dissected on ice and each region was pooled by treatment. A crude membrane preparation was prepared in lysis buffer [1% sodium dodecyl sulfate (SDS) with protease and phosphatase inhibitors] and 20 μg of protein was separated on 10–12.5% (w/v) SDS polyacrylamide mini-gels (Bio-Rad) with the exception of ClC-2 and ChAT which required 40 μg. Proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia) and blocked overnight with 5% non-fat dry milk solution. Primary antibodies for $GABA_AR$ α1 subunit (Santa Cruz, pAb, 1:1,000), $GABA_AR$ β2 subunit (Santa Cruz, pAb, 1:2,000), gephyrin (Transduction Lab., mAb, 1:250), N-cadherin (Transduction Lab., mAb, 1:10,000), α-catenin (Transduction Lab., mAb, 1:500), synaptotagmin (Transduction Lab., mAb, 1:2,000), GAD_{67} (Transduction Lab., mAb, 1:250), ClC-2 (Chemicon, pAb, 1:400), ChAT (Chemicon, pAb, 1:1000) were incubated with membranes at 22 °C for 3 h. Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Sigma, anti-goat IgG for α1, β2, ChAT; anti-mouse IgG for gephyrin, N-cadherin, α-catenin, synaptotagmin, GAD_{67}; anti-rabbit IgG for ClC-2) at 22 °C for 1 h. Proteins were detected by enhanced chemiluminescence. Scans were analyzed for density using one-dimensional

(1-D) software (Un-Scan-it 5.1, Silk Scientific) and included a step wedge (Stouffer) to insure linearity.

2.3. Statistics

Protein levels and pup body weights were analyzed using either a one sample or independent Student's *t*-test (GraphPad Prism 3), respectively. The number of independent experiments (control vs. ethanol) is denoted as 'n'. Values are reported as the mean±S.E.M. of the optical density normalized to the appropriate control for each independent experiment. Statistical significance was defined as $P<0.05$ for a two-tailed test.

3. Results

3.1. Postnatal ethanol exposure

The mean BEC 90 min after ethanol intubation on PD 6 was 302.5±6.3 mg/dl across the litters used in these experiments. This value is within the range of previous studies where binge-like ethanol treatment was administered to rat pups using either manual intubation or automated infusion methods (BEC=299±5 to 342±5 mg/dl [26–28]). The mean body weight for pups during the PD 4–9 intubation period more than doubled (controls: PD 4=10.3±0.1 g, PD 10=23.3±0.3 g; ethanol: PD 4=10.5±0.1 g, PD 10=21.3±0.2 g), although the gain for controls was slightly larger than for ethanol animals ($P<0.05$).

3.2. Effects on proteins associated with GABAergic synapses

Previously we found ethanol treatment on PDs 4–9 caused a significant reduction in whole cell currents when 300 μM GABA, a concentration near the maximum, was applied to acutely isolated MS/DB neurons on PDs 11–16 several days after ethanol treatment [26,27]. This reduced level of current suggested that fewer functional $GABA_AR$s were present on the cell surface of ethanol treated neurons. Fig. 1A shows immunoblots for $GABA_AR$ α1 and β2 subunit protein in samples of MS/DB tissue collected on PDs 13–14, a time when receptor function is reduced in individual neurons. Although there was a trend towards lower levels of β2 subunit, neither subunit was significantly reduced in MS/DB samples (Fig. 1B). Levels of GAD_{67} and ChAT were also measured (Fig. 1A), since GABAergic and cholinergic neurons represent a significant proportion of cells in MS/DB that may be impaired after perinatal ethanol exposure [30,32,43–45,58]. However, neither GAD_{67} nor ChAT was reduced after ethanol treatment (Fig. 1B) suggesting that a significant loss of these cells is unlikely. Thus in the MS/DB the previously observed significant reduction in $GABA_AR$ function at the cellular level [26,27] was not paralleled by equivalent changes in protein for receptor subunits or the GABA synthetic enzyme, GAD_{67}.

The relationship between whole cell $GABA_AR$ function and subunit level also was examined in cerebellum. Early postnatal ethanol treatment on PDs 4–9 causes well documented loss of cerebellar Purkinje cells by PD 10 [21,40,59]. However, $GABA_AR$ function on surviving Purkinje neurons is not significantly blunted on PDs 12–16, although receptor activity is reduced ~40% by PDs 25–35 [28]. Fig. 2A shows immunoblots for proteins likely involved in formation of GABAergic synapses. PD 13–14 cerebellum had a significantly reduced level of $GABA_AR$ α1 subunit (~16%) after postnatal ethanol exposure, but β2 subunit was unchanged (Fig. 2B). A 33% reduction in PD 13–14 cerebellar GAD_{67} level (Fig. 2B) was consistent with the known loss of GABAergic cerebellar Purkinje cells at this time [21,40]. Other markers associated with formation of GABAergic synapses also were examined and similarly exhibited ethanol-induced reductions. ClC-2, a voltage-gated Cl^- channel which participates in regulation of depolarizing Cl^- based signaling [55] was decreased 32% in PD 13–14 cerebellum (Fig. 2B). The synaptic vesicle protein, synaptotagmin [18], was reduced 27% by ethanol, while the adhesion molecule, N-cadherin which is associated with forming synapses and synaptic plasticity [11,29] was reduced by 11%. Finally, the intracellular anchoring/signaling partners of N-cadherin, α-catenin and β-catenin [11,60,64], were differentially affected by ethanol with α-catenin being reduced by 21% (Fig. 2B), but β-catenin showing no change (data not shown). Thus early postnatal ethanol treatment significantly reduced several GABAergic pre- and postsynaptic markers in the cerebellum on PDs 13–14, although whole cell $GABA_AR$ function on surviving Purkinje neurons was not blunted at this time [28].

Additional measurements of $GABA_AR$ α1/β2 subunits, GAD_{67}, gephyrin, ClC-2, synaptotagmin, N-cadherin, α-catenin and β-catenin were also made in brain stem, hippocampus, cerebral cortex, as well as MS/DB, to determine whether other brain areas showed the same pattern of change as was found for the cerebellum. The only other ethanol-induced changes found (see Fig. 3) were a reduction of ClC-2 in the cerebral cortex by ~37% ($P<0.05$) and a reduction in GAD_{67} and α-catenin in brain stem by ~55 and ~25%, respectively ($P<0.05$). Surprisingly, no changes were found in gephyrin, $GABA_AR$ β2 subunit protein, or in β-catenin in any of the five brain areas tested on PDs 13–14. Overall, these findings suggest a relatively selective action of ethanol to reduce GABAergic synaptic proteins in the cerebellum on PDs 13–14.

4. Discussion

The primary finding of this report is that blunted cellular level $GABA_AR$ function after an early postnatal ethanol insult [26–28] is not paralleled by tissue level decreases in

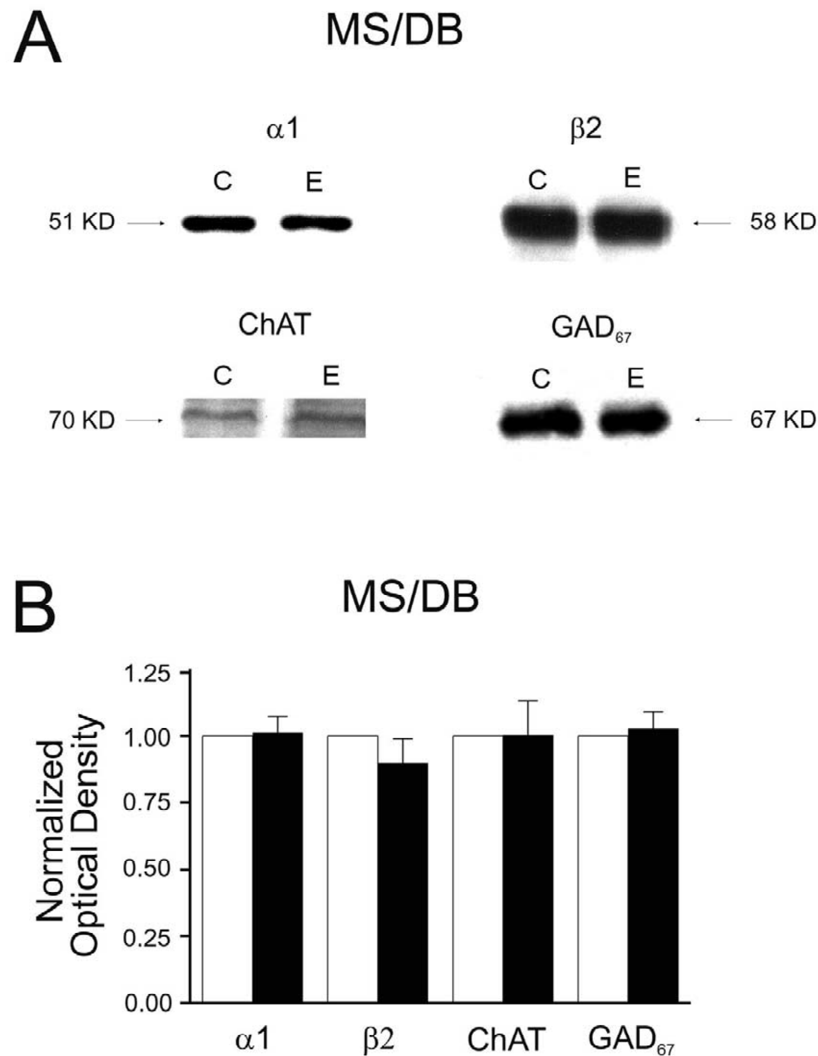

Fig. 1. Postnatal ethanol does not reduce $GABA_AR$ subunit (α1 or β2), GAD_{67} or ChAT levels in MS/DB. (A) Representative immunoblots are shown for MS/DB samples prepared from PD 13–14 animals after control (C) or ethanol (E) treatment on PDs 4–9. (B) Data resulting from quantitation of immunoblots are summarized as optical density normalized to values of the control. Control and ethanol blots were run simultaneously for each of 5–9 experiments as described in Materials and methods. No significant changes were detected.

proteins associated with GABAergic synapses on PDs 13–14. The assumption had been that ethanol arrests developmentally programmed up-regulation of $GABA_ARs$ during synaptogenesis. Whole cell $GABA_AR$-mediated Cl^- current and receptor subunit polypeptides were expected to show parallel decreases after ethanol relative to controls, because controls would continue their progressive increase with ongoing synaptogenesis. However, blunted receptor function in MS/DB neurons was not paralleled by decreases in $GABA_AR$ subunits or synapse-related peptides. In fact, the opposite condition was observed in cerebellum. $GABA_AR$ function is unchanged in Purkinje cells on PDs 13–14 after ethanol [28], while six of the GABAergic synapse-related peptides were significantly decreased at this time. Reduced levels of $GABA_AR$ α1 subunit, GAD_{67}, ClC-2, synaptotagmin, N-cadherin and α-catenin in cerebellum (Fig. 2) likely reflect the substantial cell damage occurring there which includes a loss of ~20–50% of Purkinje cells on PD 10 after early postnatal ethanol treatment [21,40]. On the other hand, the MS/DB probably does not suffer major cell loss after moderate early postnatal ethanol exposure. Moderate ethanol exposure on PDs 4–9 producing peak BECs of ~300 mg/dl does not decrease the number of MS/DB cholinergic or GABAergic neurons [32,43,44], or alter GAD_{67} or ChAT peptide levels (Fig. 1). However, more severe ethanol treatment (peak BEC of >500 mg/dl on PD 7) can cause substantial neuronal degeneration in septum on PD 8 [30], as can exposure very early in gestation [52]. Thus the primary conclusion from the present work is that changes in $GABA_AR$ subunit or synapse-related peptides found here in the cerebellum likely reflects significant neuronal loss rather than a selective functional arrest of programmed GABAergic synaptogenesis.

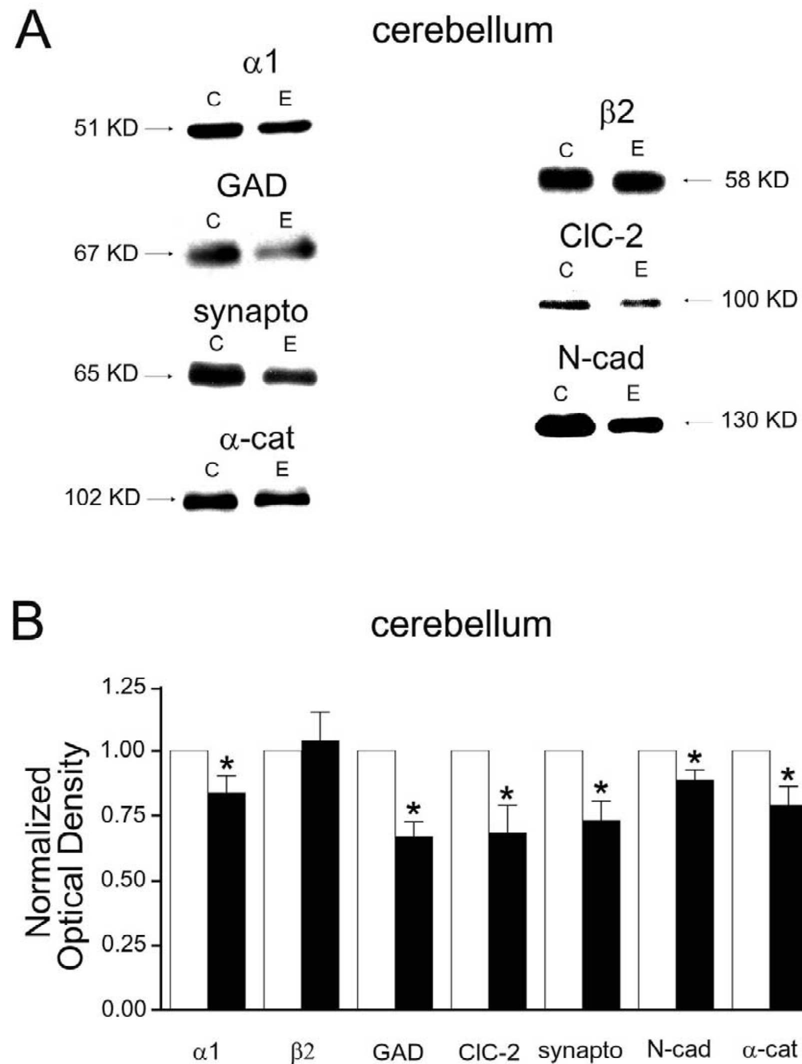

Fig. 2. Postnatal ethanol reduces $GABA_AR$ α1 subunit, GAD_{67}, ClC-2, synaptotagmin (synapto), N-cadherin (N-cad) and α-catenin (α-cat) levels in cerebellum. (A) Representative immunoblots are shown for cerebellar samples prepared from PD 13–14 animals after control (C) or ethanol (E) treatment on PDs 4–9. (B) Data resulting from quantitation of immunoblots are summarized as optical density normalized to values of the control. Control and ethanol blots were run simultaneously for each of 5–9 experiments as described in Materials and methods. $GABA_AR$ β2 subunit (β2) did not change. * $P<0.05$ for ethanol compared to control.

Why GABAergic synaptic peptide markers do not change in MS/DB when $GABA_AR$ currents are blunted by postnatal ethanol in individual MS/DB neurons [26,27] is not immediately clear. A similar lack of parallel change in $GABA_AR$ peptides and function was found in the amygdala after chronic ethanol treatment in adult animals where increased $GABA_AR$-mediated $^{36}Cl^-$ uptake in synaptoneurosomes was associated with decreased α1 and α4 subunit peptide levels [47]. However, $GABA_AR$ subunit level and function were correlated after gestational ethanol exposure in guinea pig, where increases were found in α1 and β2 subunits as well as ^{3}H-muscimol binding sites in cerebral cortex of adult offspring [3]. One reason receptor function in neurons and subunit levels in tissue may not correlate is the relative insensitivity of immunoblots which average peptide levels from all cells within a brain area which potentially diminishes neuronal level changes. In this regard, subtle changes in receptor peptides in neurons may be masked because glial cells also express a significant population of functional $GABA_ARs$ [8,12]. Glia also may be up-regulated by postnatal ethanol treatment related tissue injury [22] which could further mask a decrease in neuronal receptor subunits. It is also possible that receptor subunits in MS/DB neurons are differentially regulated such that total $GABA_AR$ number and function decrease without changing levels of α1 or β2. Although, the predominant composition of $GABA_ARs$ in MS/DB neurons is thought to switch during synaptogenesis from α2/3β2γ2 at birth to α1β2γ2 in the adult [37,63], relatively abundant message for α1/2/3, β2/3 and γ1/2 is present in adult MS/DB [15]. A reduction in other subunits besides α1 and β2 could be responsible for

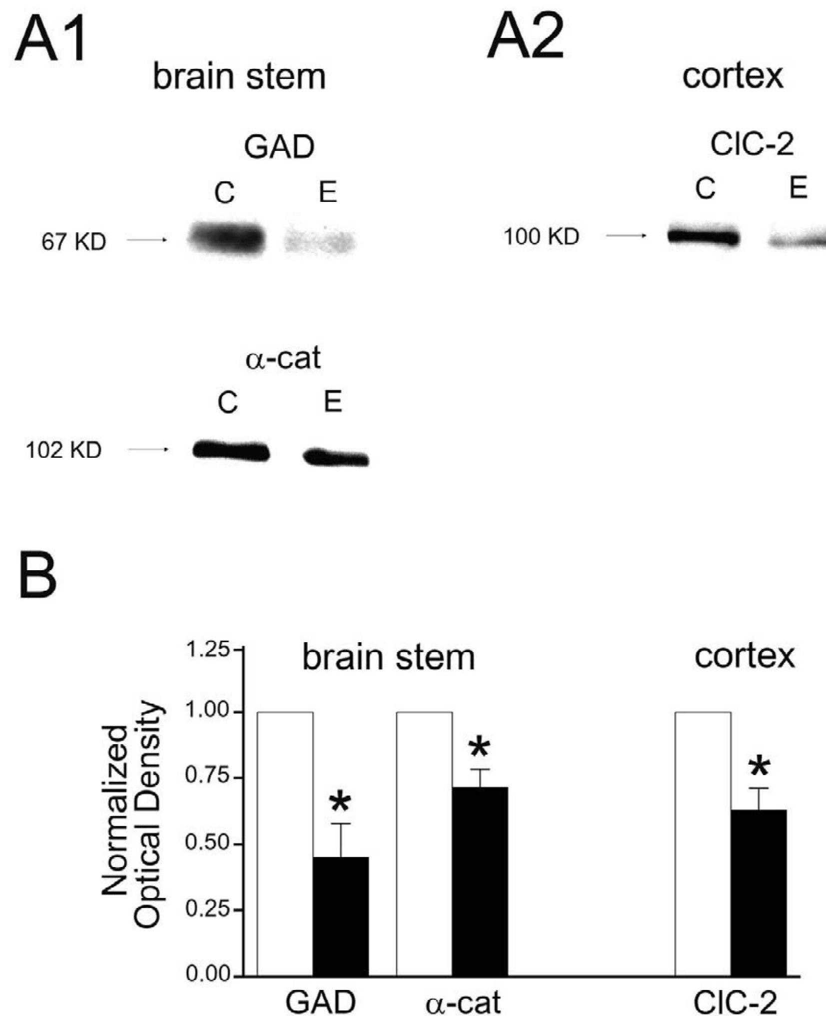

Fig. 3. Postnatal ethanol reduces GAD_{67} and/or α-catenin (α-cat) levels in brain stem and cerebral cortex. (A) Representative immunoblots are shown for brain stem or cerebral cortical samples prepared from PD 13–14 animals after control (C) or ethanol (E) treatment on PDs 4–9. (B) Data resulting from quantitation of immunoblots are summarized as optical density normalized to values of the control. Control and ethanol blots were run simultaneously for each of 4–6 experiments as described in Materials and methods. * $P<0.05$ for ethanol compared to control.

blunted whole cell $GABA_AR$ currents. Altered receptor trafficking also might explain the mismatch between function and peptide level. An increased fraction of $GABA_ARs$ might be retained in an internalized pool after postnatal ethanol so that they would not participate functionally but still contribute to peptide measures. Recycling of receptors between the plasma membrane surface and an endosomal pool via a clathrin-dependent endocytotic pathway likely plays an important role in regulating functional receptor populations [14,34,35]. Removal of surface receptors to endosomes would blunt whole cell GABA current without altering subunit peptide levels. Alternatively, ethanol-induced differences in the phosphorylation state of $GABA_ARs$ could also modify their recycling or function without changing subunit protein levels [39,46,48].

Since postnatal ethanol reduces multiple GABAergic synaptic peptides such as $GABA_AR$ α1 subunit, synaptotagmin, $GAD_{67,}$ ClC-2, N-cadherin and α-catenin in the cerebellum, supporting both loss of neurons and fewer synaptic interconnections among remaining cells, it is surprising that whole cell $GABA_AR$-currents in surviving Purkinje cells are not decreased [28]. Equally surprising is that other markers were not decreased. Neither $GABA_AR$ β2 subunit nor gephyrin, a tubulin-binding protein important for clustering of some $GABA_ARs$ at the postsynaptic density [17,36], were decreased by ethanol in any of the five brain areas studied. However, a lack of GABAergic input to isolated cultured hippocampal pyramidal or spinal cord neurons does not prevent formation of gephyrin-$GABA_AR$ clusters [38,49], suggesting that membrane clustering of $GABA_ARs$ may occur without feedback from activation of cell surface $GABA_ARs$. The lack of change in cerebellar $GABA_AR$ β2 subunit and gephyrin also might reflect increased glial contributions as damage associated with Purkinje cells loss is repaired. Ethanol did reduce ClC-2 expression both in cerebral cortex and cerebellum.

ClC-2 is thought to shunt depolarizing Cl^- gradients and passively diminish the excitatory action of $GABA_A$Rs [13,55]. This reduction of ClC-2 may be consistent with an ethanol-induced distortion of GABAergic signaling during synaptogenesis. Ethanol does exert a small but consistent acute inhibitory action on $GABA_A$R activity in both MS/DB and Purkinje neurons over PDs 4–9 [27,28], which might disrupt other protein interactions essential for synaptogenesis. Possibly cerebellar damage from binge-like postnatal ethanol intubation might be offset by pharmacologically enhancing activation of $GABA_A$Rs with agonists, a manipulation that has been shown to reverse ethanol inhibition of neurite outgrowth in cultured cholinergic neurons from chick embryo [56].

Finally, ethanol could influence appropriate synapse formation by distorting regulation of cell surface adhesion molecules such as the cadherins, immunoglobulin (Ig) cell adhesion molecules (CAMs) or integrins that participate in formation of synaptic junctions during target recognition, synaptogenesis and synaptic plasticity [53]. PD 4–9 ethanol treatment did reduce N-cadherin in cerebellum and its intracellular binding partner, α-catenin in brain stem and cerebellum (Figs. 2 and 3). Cerebellar granule cells in dissociated culture still extend neurites in the presence of ethanol on an N-cadherin substrate, although neurite outgrowth is impaired when the substrate is L1 [6]. Prenatal ethanol exposure can distort NCAM peptide levels in cerebral cortex over PDs 5–30 [42], while neuronal NCAM staining in cerebral cortical slice cultures is distorted after 48 h ethanol exposure [25]. Together these findings suggest that ethanol may disrupt appropriate synapse formation by interfering directly or indirectly with a number of functional molecular interactions involved in cell–cell association.

Acknowledgements

The authors are grateful to Drs. R.C. Burghardt and D.C. Zawieja for sharing their expertise and the use of their microscopes and imaging software and to Dr. Ursula Winzer-Serhan for thoughtful comments on the manuscript. We thank Dr. James R. West for providing the synthetic milk. We also thank Jason L. Acevedo, Dustin W. DuBois, Anitra M. Guillory and Kelly R. Smith for preparing the animals and Larry S. Cooke for his excellent technical assistance. This work was supported by NIH grant AA 12386 to G.D.F.

References

[1] A.M. Allan, H. Wu, L.L. Paxton, D.D. Savage, Prenatal ethanol exposure alters the modulation of the γ-aminobutyric $acid_A$ receptor-gated chloride ion channel in adult rat offspring, J. Pharmacol. Exp. Ther. 284 (1998) 250–257.

[2] C.D.C. Bailey, J.F. Brien, J.N. Reynolds, Altered $GABA_A$-benzodiazepine receptor number and pharmacology in the adult guinea pig cerebral cortex after chronic prenatal ethanol exposure, Alcohol. Clin. Exp. Res. 23 (1999) 1816–1824.

[3] C.D.C. Bailey, J.F. Brien, J.N. Reynolds, Chronic prenatal ethanol exposure increases $GABA_A$ receptor subunit protein expression in the adult guinea pig cerebral cortex, J. Neurosci. 21 (2001) 4381–4389.

[4] J.L. Barker, T. Behar, Y.X. Li, Q.Y. Liu, W. Ma, D. Maric, I. Maric, A.E. Schaffner, R. Serafini, S.V. Smith, R. Somogyi, J.Y. Vautrin, X.L. Wen, H. Xian, GABAergic cells and signals in CNS development, Perspect. Dev. Neurobiol. 5 (1998) 305–322.

[5] E.A. Barnard, P. Skolnick, R.W. Olsen, H. Mohler, W. Sieghart, G. Biggio, C. Braestrup, A.N. Bateson, S.Z. Langer, International Union of Pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function, Pharmacol. Rev. 50 (1998) 291–313.

[6] C.F. Bearer, A.R. Swick, M.A. O'Riordan, G.H. Cheng, Ethanol inhibits L1-mediated neurite outgrowth in postnatal rat cerebellar granule cells, J. Biol. Chem. 274 (1999) 13264–13270.

[7] H.C. Becker, J.L. Diaz-Granados, C.L. Randall, Teratogenic actions of ethanol in the mouse: a minireview, Pharmacol. Biochem. Behav. 55 (1996) 501–513.

[8] L.K. Bekar, R. Jabs, W. Walz, $GABA_A$ receptor agonists modulate K^+ currents in adult hippocampal glial cells in situ, Glia 26 (1999) 129–138.

[9] B. Belhage, G.H. Hansen, L. Elster, A. Schousboe, Effects of γ-aminobutyric acid (GABA) on synaptogenesis and synaptic function, Perspect. Dev. Neurobiol. 5 (1998) 235–246.

[10] Y. Ben-Ari, R. Khazipov, X. Leinekugel, O. Caillard, J.L. Gaiarsa, $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'menage a trois', Trends Neurosci. 20 (1997) 523–529.

[11] D.L. Benson, H. Tanaka, N-cadherin redistribution during synaptogenesis in hippocampal neurons, J. Neurosci. 18 (1998) 6892–6904.

[12] M. Bureau, J. Laschet, M. Bureauheeren, B. Hennuy, A. Minet, P. Wins, T. Grisar, Astroglial cells expression large amounts of $GABA_A$ receptor proteins in mature brain, J. Neurochem. 65 (1995) 2006–2015.

[13] G.H. Clayton, K.J. Staley, C.L. Wilcox, G.C. Owens, R.L. Smith, Developmental expression of ClC-2 in the rat nervous system, Dev. Brain Res. 108 (1998) 307–318.

[14] C.N. Connolly, J.T. Kittler, P. Thomas, J.M. Uren, N.J. Brandon, T.G. Smart, S.J. Moss, Cell surface stability of γ-aminobutyric acid type A receptors—Dependence on protein kinase C activity and subunit composition, J. Biol. Chem. 274 (1999) 36565–36572.

[15] H.E. Criswell, P.E. Simson, D.J. Knapp, L.L. Devaud, T.J. McCown, G.E. Duncan, A.L. Morrow, G.R. Breese, Effect of zolpidem on γ-aminobutyric acid (GABA)-induced inhibition predicts the interaction of ethanol with GABA on individual neurons in several rat brain regions, J. Pharmacol. Exp. Ther. 273 (1995) 526–536.

[16] C.D. Driscoll, A.P. Streissguth, E.P. Riley, Prenatal alcohol exposure: comparability of effects in humans and animal models, Neurotoxicol. Teratol. 12 (1990) 231–237.

[17] C. Essrich, M. Lorez, J.A. Benson, J.M. Fritschy, B. Luscher, Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin, Nat. Neurosci. 1 (1998) 563–571.

[18] R. Fernandez-Chacon, A. Konigstorfer, S.H. Gerber, J. Garcia, M.F. Matos, C.F. Stevens, N. Brose, J. Rizo, C. Rosenmund, T.C. Südhof, Synaptotagmin I functions as a calcium regulator of release probability, Nature 410 (2001) 41–49.

[19] J.M. Fritschy, J. Paysan, A. Enna, H. Mohler, Switch in the expression of rat $GABA_A$-receptor subtypes during postnatal development: an immunohistochemical study, J. Neurosci. 14 (1994) 5302–5324.

[20] K. Ganguly, A.F. Schinder, S.T. Wong, M.M. Poo, GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition, Cell 105 (2001) 521–532.

[21] C.R. Goodlett, A.D. Pearlman, K.R. Lundahl, Binge neonatal alcohol intubations induce dose-dependent loss of Purkinje cells, Neurotoxicol. Teratol. 20 (1998) 285–292.

[22] C.R. Goodlett, S.D. Peterson, K.R. Lundahl, A.D. Pearlman, Binge-like alcohol exposure of neonatal rats via intragastric intubation induces both Purkinje cell loss and cortical astrogliosis, Alcohol. Clin. Exp. Res. 21 (1997) 1010–1017.

[23] S.H. Han, B.A. McCool, D. Murchison, S.S. Nahm, A.R. Parrish, W.H. Griffith, Single-cell RT-PCR detects shifts in mRNA expression profiles of basal forebrain neurons during aging, Mol. Brain Res. 98 (2002) 67–80.

[24] J.H. Hannigan, R.F. Berman, Amelioration of fetal alcohol-related neurodevelopmental disorders in rats: exploring pharmacological and environmental treatments, Neurotoxicol. Teratol. 22 (2000) 103–111.

[25] K. Hirai, H. Yoshioka, M. Kihara, K. Hasegawa, T. Sawada, S. Fushiki, Effects of ethanol on neuronal migration and neural cell adhesion molecules in the embryonic rat cerebral cortex: a tissue culture study, Dev. Brain Res. 118 (1999) 205–210.

[26] S.-H. Hsiao, J.L. Acevedo, D.W. DuBois, K.R. Smith, J.R. West, G.D. Frye, Early postnatal intubation blunts $GABA_A$ receptor up-regulation and modifies 3α-hydroxy-5α-pregnan-20-one sensitivity in rat MS/DB neurons, Dev. Brain Res. 130 (2001) 25–40.

[27] S.-H. Hsiao, J.C. Mahoney, J.R. West, G.D. Frye, Development of $GABA_A$ receptors on medial septum/diagonal band (MS/DB) neurons after postnatal ethanol exposure, Brain Res. 810 (1998) 100–113.

[28] S.-H. Hsiao, J.R. West, J.C. Mahoney, G.D. Frye, Postnatal ethanol exposure blunts upregulation of $GABA_A$ receptor currents in Purkinje neurons, Brain Res. 832 (1999) 124–135.

[29] G.W. Huntley, D.L. Benson, Neural (N)-cadherin at developing thalamocortical synapses provides an adhesion mechanism for the formation of somatopically organized connections, J. Comp. Neurol. 407 (1999) 453–471.

[30] C. Ikonomidou, P. Bittigau, M.J. Ishimaru, D.F. Wozniak, C. Koch, K. Genz, M.T. Price, V. Stefovska, F. Horster, T. Tenkova, K. Dikranian, J.W. Olney, Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome, Science 287 (2000) 1056–1060.

[31] L. Janiri, G. Gobbi, A.M. Persico, M. Santarelli, D. Minciacchi, E. Tempesta, Alterations of neocortical neuronal responses to acetylcholine and GABA in rats born to alcohol-dependent mothers, Alcohol Alcohol. 29 (1994) 611–619.

[32] T.B. Johnson, C.R. Goodlett, Binge alcohol exposure on postnatal day 7–9 in rats reduces the relative number of parvalbumin- and ChAT-immunoreactive neurons in the medial septum, Soc. Neurosci. Abstr. 23 (1997) 1863.

[33] S.B. Kater, L.R. Mills, Regulation of growth cone behavior by calcium, J. Neurosci. 11 (1991) 891–899.

[34] J.T. Kittler, P. Delmas, J.N. Jovanovic, D.A. Brown, T.G. Smart, S.J. Moss, Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons, J. Neurosci. 20 (2000) 7972–7977.

[35] J.T. Kittler, J.F. Wang, C.N. Connolly, S. Vicini, T.G. Smart, S.J. Moss, Analysis of $GABA_A$ receptor assembly in mammalian cell lines and hippocampal neurons using γ2 subunit green fluorescent protein chimeras, Mol. Cell. Neurosci. 16 (2000) 440–452.

[36] M. Kneussel, J.H. Brandstatter, B. Laube, S. Stahl, U. Muller, H. Betz, Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice, J. Neurosci. 19 (1999) 9289–9297.

[37] D.J. Laurie, W. Wisden, P.H. Seeburg, The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development, J. Neurosci. 12 (1992) 4151–4172.

[38] S. Levi, D. Chesnoy-Marchais, W. Sieghart, A. Triller, Synaptic control of glycine and $GABA_A$ receptors and gephyrin expression in cultured motoneurons, J. Neurosci. 19 (1999) 7434–7449.

[39] Y.F. Lin, T.P. Angelotti, E.M. Dudek, M.D. Browning, R.L. Macdonald, Enhancement of recombinant α1β1γ2L γ-aminobutyric $acid_A$ receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both β1 and γ2L subunits, Mol. Pharmacol. 50 (1996) 185–195.

[40] B.L. Marcussen, C.R. Goodlett, J.C. Mahoney, J.R. West, Developing rat Purkinje cells are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis, Alcohol 11 (1994) 147–156.

[41] R.M. McKernan, P.J. Whiting, Which $GABA_A$-receptor subtypes really occur in the brain?, Trends Neurosci. 19 (1996) 139–143.

[42] R. Minana, E. Climent, D. Barettino, J.M. Segui, J. Renau-Piqueras, C. Guerri, Alcohol exposure alters the expression pattern of neural cell adhesion molecules during brain development, J. Neurochem. 75 (2000) 954–964.

[43] J.J. Mitchell, M. Pavia, M.B. Heaton, Effect of neonatal ethanol exposure on parvalbumin-expressing GABAergic neurons of the rat medial septum and cingulate cortex, Alcohol 21 (2000) 49–57.

[44] D.B. Moore, P. Lee, M. Paiva, D.W. Walker, M.B. Heaton, Effects of neonatal ethanol exposure on cholinergic neurons of the rat medial septum, Alcohol 15 (1998) 219–226.

[45] D.B. Moore, A.C. Ruygrok, D.W. Walker, M.B. Heaton, Effects of prenatal ethanol exposure on parvalbumin-expression GABAergic neurons in the adult rat medial septum, Alcohol. Clin. Exp. Res. 21 (1997) 849–856.

[46] S.J. Moss, G.H. Gorrie, A. Amato, T.G. Smart, Modulation of $GABA_A$ receptors by tyrosine phosphorylation, Nature 377 (1995) 344–348.

[47] S. Papadeas, A.C. Grobin, A.L. Morrow, Chronic ethanol consumption differentially alters $GABA_A$ receptor α1 and α4 subunit peptide expression and $GABA_A$ receptor-mediated $^{36}Cl^-$ uptake in mesocorticolimbic regions of rat brain, Alcohol. Clin. Exp. Res. 25 (2001) 1270–1275.

[48] N.M. Porter, R.E. Twyman, M.D. Uhler, R.L. Macdonald, Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons, Neuron 5 (1990) 789–796.

[49] A. Rao, E.M. Cha, A.M. Craig, Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons, J. Neurosci. 20 (2000) 8344–8353.

[50] C. Rivera, J. Voipio, J.A. Payne, E. Ruusuvuori, H. Lahtinen, K. Lamsa, U. Pirvola, M. Saarma, K. Kaila, The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation, Nature 397 (1999) 251–255.

[51] P.D. Sampson, A.P. Streissguth, F.L. Bookstein, R.E. Little, S.K. Clarren, P. Dehaene, J.W. Hanson, J.M. Graham, Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder, Teratology 56 (1997) 317–326.

[52] U.B. Schambra, J.M. Lauder, P. Petrusz, K.K. Sulik, Development of neurotransmitter systems in the mouse embryo following acute ethanol exposure: a histological and immunocytochemical study, Int. J. Dev. Neurosci. 8 (1990) 507–522.

[53] L. Shapiro, D.R. Colman, The diversity of cadherins and implications for a synaptic adhesive code in the CNS, Neuron 23 (1999) 427–430.

[54] W. Sieghart, Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes, Pharmacol. Rev. 47 (1995) 181–234.

[55] R.L. Smith, G.H. Clayton, C.L. Wilcox, K.W. Escudero, K.J. Staley, Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: a potential mechanism for cell-specific modulation of postsynaptic inhibition, J. Neurosci. 15 (1995) 4057–4067.

[56] P.E. Spoerri, N. Srivastava, A. Vernadakis, Role of $GABA_A$ receptors in the GABA attenuation of ethanol neurotoxicity, J. Neurosci. Res. 44 (1996) 499–506.

[57] K. Staley, R. Smith, J. Schaack, C. Wilcox, T.J. Jentsch, Alteration of $GABA_A$ receptor function following gene transfer of the CLC-2 chloride channel, Neuron 17 (1996) 543–551.

[58] D.J. Swanson, L. Tonjes, M.A. King, D.W. Walker, M.B. Heaton,

Influence of chronic prenatal ethanol on cholinergic neurons of the septohippocampal system, J. Comp. Neurol. 364 (1996) 104–112.
[59] J.D. Thomas, C.R. Goodlett, J.R. West, Alcohol-induced Purkinje cell loss depends on developmental timing of alcohol exposure and correlates with motor performance, Dev. Brain Res. 105 (1998) 159–166.
[60] T. Uemura, The cadherin superfamily at the synapse: more members, more missions, Cell 93 (1998) 1095–1098.
[61] J.R. West, Use of pup in a cup model to study brain development, J. Nutr. 123 (1993) 382–385.
[62] J.R. West, K.M. Hamre, D.R. Pierce, Delay in brain growth induced by alcohol in artificially reared rat pups, Alcohol 1 (1984) 213–222.
[63] W. Wisden, D.J. Laurie, H. Monyer, P.H. Seeburg, The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon, J. Neurosci. 12 (1992) 1040–1062.
[64] T. Yagi, M. Takeichi, Cadherin superfamily genes: functions, genomic organization, and neurologic diversity, Genes Dev. 14 (2000) 1169–1180.