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Differential Effects of Ethanol on c-Jun N-Terminal Kinase, 14-3-3 Proteins, and Bax in Postnatal Day 4 and Postnatal Day 7 Rat Cerebellum

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Abstract

These studies investigated ethanol effects on upstream cellular elements and interactions which contribute to Bax-related apoptosis in neonatal rat cerebellum at ages of peak ethanol sensitivity (postnatal day 4 [P4]), compared to later ages of relative resistance (P7). Analyses were made of basal levels of the pro-apoptotic c-jun N-terminal kinase (JNK), Bax, and the 14-3-3 anchoring proteins, as well as the responsiveness of these substances to ethanol at P4 versus P7. Dimerization of Bax with 14-3-3 was also investigated at the two ages following ethanol treatment, a process which sequesters Bax in the cytosol, thus inhibiting its mitochondrial translocation and disruption of the mitochondrial membrane potential. Cultured cerebellar granule cells were used to examine the protective potential of JNK inhibition on ethanol-mediated cell death. Basal levels of JNK were significantly higher at P4 than P7, but no differences in the other proteins were found. Activated JNK, and cytosolic and mitochondrially-translocated Bax were increased in P4 but not P7 animals following ethanol exposure, while protective 14-3-3 proteins were increased only at P7. Ethanol treatment resulted in decreases in Bax:14-3-3 heterodimers at P4, but not at P7. Inhibition of JNK activity in vitro provided partial protection against ethanol neurotoxicity. Thus, differential temporal vulnerability to ethanol in this CNS region correlates with differences in both levels of apoptosis-related substances (e.g., JNK), and differential cellular responsiveness, favoring apoptosis at the most sensitive age and survival at the resistant age. The upstream elements contributing to this vulnerability can be targets for future therapeutic strategies.

Keywords

Ethanol; fetal alcohol syndrome; apoptosis; cerebellum

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1. Introduction

The developing central nervous system (CNS) is exquisitely sensitive to ethanol, with early exposure leading to an array of anomalies, collectively known as fetal alcohol spectrum disorder (FASD). At the far end of this spectrum, is the fetal alcohol syndrome (FAS), a devastating condition first recognized in the 1970s, characterized by behavioral, cognitive and intellectual impairment, with widely distributed underlying neuropathology (Jones and Smith, 1973). Despite repeated warnings about the dangers of developmental ethanol exposure, the incidence of FAS/FASD continues to escalate. In the developing CNS, ethanol leads to a marked depletion of neuronal populations, with certain regions being selectively sensitive. Within these regions, critical periods have been empirically defined in animal models. In developing cerebellum, for example, in the rodent FASD model, the period of maximal ethanol vulnerability is during the first postnatal week, with a peak period of sensitivity at postnatal days 4–5 (P4–5). Even brief exposure during this peak period leads to marked loss of cerebellar Purkinje and granule cells (Goodlett et al., 1990). Only slightly later within the neonatal period, this region is able to withstand ethanol insult, with relatively minimal neuronal damage (P7–8; Bonthius and West, 1991; Goodlett and Eilers, 1997; Pierce et al., 1999). Research concerned with defining molecular mechanisms underlying ethanol-mediated cell loss suggests that such cellular depletion is an apoptotic process, with strong involvement of the Bcl-2 survival-regulatory gene family (Moore et al., 1999; Olney et al., 2001; 2002; Inoue et al., 2002; Heaton et al., 2003a;b;c; Ge et al., 2004; Nowoslawski et al., 2005; Lee et al., 2008). Within this family, the pro-apoptotic Bax protein appears to be critical to ethanol-induced disruptions (Young et al., 2003; Heaton et al., 2006). The role of this molecule in ethanol neurotoxicity was demonstrated most effectively in studies of Bax gene-deleted animals, in which cell loss following neonatal ethanol treatment was essentially eliminated in several CNS regions, even when exposure is rendered during their peak period of sensitivity (e.g., P7 cortex, thalamus and hippocampus [Young et al., 2003]; P4 cerebellum [Heaton et al., 2006]).

While Bax is important in the apoptosis cascade initiated by ethanol, many of the molecular collaborators within this pathway have not yet been elucidated. For the present study, we investigated the possible role of the 14-3-3 proteins in ethanol neurotoxicity. This family of highly conserved eukaryotic regulatory molecules serves a variety of roles, functioning by binding and modulating activities of numerous signaling proteins (Masters and Fu, 2001). Within the apoptotic pathway, the 14-3-3 proteins provide protective cytosolic anchors for both Bax and Bad, abrogating their apoptotic activities (Watanabe et al., 1994; Nomura et al., 2003; Tsuruta et al 2004). There are ten recognized 14-3-3 isoforms, with at least five known to bind Bax (sigma, eta, epsilon, zeta and theta; Nomura et al., 2003; Tsuruta et al., 2004; Gao et al., 2005). The 14-3-3 proteins are phosphorylated by the c-jun N-terminal kinase (JNK), which acts to weaken Bax:14-3-3 binding, freeing Bax to travel to the mitochondria. At the mitochondria, Bax participates in the disturbance of the membrane potential, leading to release of cytochrome-c, and triggering the apoptotic cascade.

For the present study, then, we investigated cellular elements and interactions which contribute to Bax-related apoptosis in developing cerebellum, and the possible differential effects of ethanol on these processes at the age of maximal sensitivity (P4), compared to the later age of relative resistance (P7). The following studies were conducted: (1) Analyses of baseline levels of JNK, 14-3-3, and Bax proteins at the age of peak vulnerability and the later less sensitive age; (2) assessment of activation of JNK, expression of 14-3-3, and subcellular localization of Bax following ethanol exposure at the two ages; (3) examination of the effects of ethanol exposure on Bax:14-3-3 binding at both P4 and P7; and (4) determination of the importance of JNK activation to the ethanol-mediated apoptotic process within developing cerebellum, using an in vitro approach.

2. Results

2.1. Blood Ethanol Concentrations

The blood ethanol concentrations (BECs) of P4 and P7 animals, measured 2 hours following termination of vapor inhalation, averaged 273 ± 3.92 mg/dl in the P4 pups, and 279 ± 3.42 mg/dl in the P7 pups. We have previously shown that BECs measured at this interval following vapor inhalation represent peak levels (Heaton et al., 2003a).

2.2. ELISA Analyses of Basal Expression of JNK, 14-3-3 and Bax Proteins in P4 and P7 Cerebellum

Analyses of baseline levels of JNK, the 14-3-3 proteins, and Bax at P4 and P7 were made in order to determine whether differential expression levels at the vulnerable and resistant ages may contribute to the differential ethanol sensitivity at the two ages. These analyses were made via enzyme-linked immunosorbent assays (ELISA). These analyses of the JNK kinase, using antibodies which recognized JNK independent of its phosphorylation status, revealed significantly greater expression levels at P4 compared to P7, as measured by the Student's t-test ($P4 = 1.5X$ that measured at P7; $t=8.037$; $p=0.0013$; Figure 1). The 14-3-3 ELISA analyses, using pan-14-3-3 antibodies which recognized all of the 14-3-3 isoforms, showed no differences in basal levels of these proteins between the two ages (data not shown). Similarly, no differences were found in basal levels of Bax protein at the two ages (data not shown).

2.3. Western Blot Analyses of Activated JNK, 14-3-3 Proteins, and Cytosolic and Mitochondrial Bax, in Neonatal Cerebellum Following Ethanol Exposure at P4 and P7

Comparative Western blot analyses of activated (i.e., phosphorylated) JNK (pJNK), 14-3-3 proteins, and Bax were made following ethanol exposure via vapor inhalation in order to detect possible differential responsiveness at the two developmental ages. There are ten isoforms within the JNK family, generated by alternative splicing of transcripts of the JNK 1, 2 and 3 genes (Tsuruta et al., 2004). Each gene is expressed as 46kDa or 55kDa proteins, depending on the processing of the corresponding mRNA coding region. These two isoforms appear to be functionally similar, although there is some evidence that the 46kDa isoform may be responsible for much of the kinase activity (Ip and Davis, 1998; Pimienta and Pascual, 2007). In the neonatal cerebellar tissue in the present study, this lower molecular weight isoform was by far the predominant one, and was chosen for analysis. The two-way ANOVA applied to the pro-apoptotic pJNK data revealed a significant effect of treatment ($F [1,27] = 59.89$, $p < 0.0001$) and age ($F [1,27] = 83.09$, $p < 0.0001$), as well as a significant treatment X age interaction ($F [1,27] = 47.34$, $p < 0.0001$). Individual comparisons via the Fisher Protected Least Significant Difference (PLSD) posthoc test indicated a striking enhancement of JNK in the P4 ethanol-exposed cerebella, to 3.19X that measured in controls ($p=0.0047$), but the small increase of this kinase found in the P7 animals (to 1.19X control values) did not reach statistical significance. These data are presented in Figure 2A, with representative Western blots of this protein shown in Figure 2B. Levels of cerebellar cyclophilin-40 protein, used as a loading control, and to assess the generality of the ethanol effects, were similar for control and ethanol conditions, at both ages, in this and the following experiments. Representative Western blots of cyclophilin-40 are shown in Figure 2C.

As noted above, there are multiple 14-3-3 isoforms which bind the Bax protein. For our analyses, a pan-14-3-3 antibody was used, which recognizes all isoforms. The two-way ANOVA applied to these analyses following P4 and P7 ethanol exposure indicated a significant effect of treatment ($F [1,14] = 7.05$; $p=0.018$), age ($F [1,14] = 5.22$, $p < 0.038$), and a treatment X age interaction ($F [1,14] = 5.74$, $p < 0.031$). Post hoc evaluations indicated that

these protective proteins were again differentially affected by ethanol treatment at the two developmental ages, with a significant increase seen following ethanol treatment at the ethanol-resistant age (P7; to 1.37X control levels; $p=0.0145$), but not at the more vulnerable age (P4; Figure 3).

Analyses of Western blots of cytosolic Bax protein revealed a robust elevation in the ethanol-treated cerebella at the P4 age, to 2.53X that measured in control animals (Figure 4). The two-way ANOVA applied to this data showed main effects of treatment ($F [1,81] = 15.06$, $p=0.0002$) and age ($F [1,81] = 11.07$, $p = 0.0013$), and a significant treatment X age interaction ($F [1,81] = 9.72$, $p=0.0025$). Individual comparisons via the PLSD posthoc test indicated a significant difference in this apoptosis effector between the ethanol and controls at the age of peak ethanol sensitivity (P4; $p=0.0005$), while the slight elevation at the age of relative ethanol resistance (P7) was not significant. Similar analyses of mitochondrially translocated Bax also revealed main effects of treatment ($F [1,16] = 195.45$, $p < 0.0001$) and age ($F [1,16] = 360.98$, $p < 0.0001$), and a significant treatment X age interaction ($F [1,16] = 330.28$, $p < 0.0001$). Post hoc comparisons showed a significant increase in mitochondrially localized Bax in the P4 ethanol-treated animals, to 1.52X that seen in controls ($p < 0.0001$; Figure 5). In the P7 animals, however, similar ethanol treatment resulted in a very small but statistically significant *decrease* in mitochondrial Bax, to 0.93X control levels ($p=0.016$; Figure 5).

2.4. ELISA Analyses of Bax:14-3-3 Interactions in Neonatal Cerebellum Following Ethanol Exposure at P4 and P7

Protein-protein heterodimeric interactions between Bax and 14-3-3, important for sequestering Bax in the cytosol and inhibiting its migration to the mitochondrial membrane, were assessed by our ELISA-based approach. The analyses of these assessments, via the two-way ANOVA, revealed a significant main effect of treatment ($F [1,92] = 43.41$; $p < 0.0001$), age ($F [1,92] = 6.72$; $p=0.0111$), and a significant treatment X age interaction ($F [1,92] = 14.07$; $p = 0.0003$). Individual post hoc comparisons indicated differential responsiveness to ethanol at the two developmental ages: At P4, the peak period of ethanol sensitivity, acute treatment resulted in a significant decrease in the presence of Bax:14-3-3 dimers, to 0.70X control levels ($p < 0.0001$). Conversely, in the P7 animals, the small decline in these interactions following similar treatment did not reach statistical significance. These data are presented in Figure 6.

2.5. Analyses of Neuronal Survival of Cerebellar Granule Cells Co-Cultured with Ethanol and JNK Inhibitor

P8 cerebellar granule cell preparations were used to determine whether inhibition of the JNK kinase would reduce ethanol toxicity within this developing neuronal population. For these analyses, granule cell cultures were exposed to ethanol with and without 1,9-pyrazoloanthrone (SP600125), a selective, small molecule, cell-permeable, reversible anthracycline JNK inhibitor. Three ethanol concentrations were used for these analyses: 100, 250 and 400mg/dl. These concentrations represent moderate-to-relatively high levels, and are well within the range seen both in awake humans following binge consumption, and in animal studies of ethanol toxicity (e.g., Lindblad and Olsson, 1976; Young et al., 2003). The JNK inhibitor was applied at concentrations of 200 nM, 10 μ M, 20 μ M, and 30 μ M, in an attempt to define the effective concentration. Experimental conditions (i.e., ethanol and JNK inhibitor) were applied either simultaneously, or the cells were pretreated with the JNK inhibitor for a period of one hour. The ethanol exposure interval in both cases was 24 hours, followed by analyses of survival via the MTT assay. This assay relies on the cleavage of the tetrazolium salt MTT by the mitochondrial enzyme succinate-dehydrogenase, a conversion which can be performed only in cells with functional mitochondria. The assay is thus a

reliable measure of survival in non-proliferative populations such as cerebellar granule cells (Oberdoerster and Rabin, 1999). Our initial analyses found, somewhat surprisingly, no significant differences in the neuroprotective potential of the four inhibitor concentrations, and none of these concentrations were neurotoxic, in agreement with previous studies (Bennett et al., 2001; Lee and Shukla, 2005). Figure 7 depicts comparative survival across the four inhibitor concentrations, with data from the highest ethanol concentration shown (similar effects were found with the other ethanol concentrations; data not shown). This lack of an apparent dose-response effect with respect to this population may be reflective of a high degree of sensitivity, with maximal enhancement of survival achieved with the lowest concentration used. In addition, no protective advantage was afforded by pretreatment (data not shown). Therefore, concentration/treatment data were combined for further analyses. The ANOVA applied to these combined data indicated a significant effect of condition ($F [7, 280] = 69.62, p < 0.0001$). Post hoc analyses indicated that 24-hour survival in these neuronal preparations was not altered by inclusion of the JNK inhibitor in the medium, at any of the concentrations used. Supplementation with ethanol, however, resulted in significantly diminished survival compared to controls at 24 hours post-treatment: In cultures with 100mg/dl ethanol, survival decreased by 14%, with 250mg/dl, survival was decreased 27%, and with 400mg/dl ethanol, neuronal survival was 43% lower than that in control cultures ($p < 0.0001$ in each instance). Inclusion of the JNK inhibitor in the ethanol cultures resulted in survival that significantly exceeded that seen in the cultures with ethanol alone ($p < 0.0005$ with each ethanol concentration), and in cultures with the lowest ethanol concentration (100mg/dl), this inhibition resulted in survival levels that did not differ significantly from that found in control cultures.

With the higher ethanol concentrations, however, while JNK inhibition significantly enhanced neuronal survival, complete rescue was not seen. With the 250 mg/dl ethanol concentration, inclusion of the inhibitor resulted in cell loss of only 11% compared to 27% with ethanol alone, and with 400mg/dl ethanol, neuronal loss of 22% was found when ethanol and JNK inhibitor were combined, compared to 43% in this ethanol concentration alone. With both ethanol levels, however, survival in the presence of the inhibitor differed significantly from controls ($p < 0.0001$ with both concentrations). These results are summarized in Figure 8.

3. Discussion

This series of studies investigated critical upstream events which contribute to ethanol-induced Bax neurotoxicity. This pro-apoptotic protein, acting via the intrinsic pathway, has been shown to play a key role in mediating neuronal loss following developmental ethanol exposure (Young et al., 2003; Heaton et al., 2006). The present study focused on several interactive events which are critical to Bax-mediated apoptosis. These events were assessed at the age of maximal ethanol sensitivity in neonatal cerebellum (postnatal day 4), and at a slightly later age which has been shown to be relatively ethanol-resistant (postnatal day 7). The studies were designed to determine whether differences in ethanol sensitivity are paralleled by differences in basal levels of key intermediaries, or differential responsiveness of these substances to ethanol exposure. Assessments made at the two developmental ages included analyses of basal levels and ethanol-induced alterations in the pro-apoptotic c-jun N-terminal kinase (JNK), the 14-3-3 proteins, and Bax; the effects of ethanol treatment on the integrity of protective Bax:14-3-3 complexes; and the capacity of JNK inhibition, in a cell culture system, to protect against ethanol neurotoxicity. Collectively, these studies have shown that ethanol elicits strikingly different alterations in Bax, JNK, 14-3-3 proteins, and Bax:14-3-3 interactions at the two ages, with the changes produced on P4, the maximally vulnerable age, clearly favoring an apoptotic outcome. These changes include elevations in cytosolic and mitochondrially-translocated Bax, increases in activated JNK, and decreases in

Bax:14-3-3 complexes. In addition, basal levels of JNK at this age greatly exceed those measured at the resistant age. Conversely, on P7, the ethanol-resistant age in this developing region, no changes were found in JNK, mitochondrial Bax *decreased* slightly, and the Bax:14-3-3 dimers were unchanged as a function of ethanol treatment. The protective 14-3-3 proteins, however, were significantly *increased* by ethanol at this age. At this later age, then, ethanol responsiveness was either protective (enhanced 14-3-3 availability) or neutral (no spike in Bax or JNK, no enhanced liberation of Bax from 14-3-3 sequestration). Thus, this study reveals differential responsiveness of key upstream apoptosis-related molecules which likely contribute significantly to the differential ethanol sensitivity at these two developing ages. The following sections consider these findings in the context of prior investigations.

3.1. The Role of Bax in Developmental Ethanol Neurotoxicity

Bax, a pro-apoptotic member of the Bcl-2 survival-regulatory gene family, is normally a 21kDa monomeric cytosolic protein, and in healthy resting cells is sequestered in the cytosol by the 14-3-3 anchoring proteins (Gao et al., 2005; Roucou et al., 2005). Following JNK phosphorylation of 14-3-3, Bax is released from this cytosolic anchor, and translocates to the mitochondrial membrane, where it homodimerizes, and heterodimerizes with apoptosis antagonists Bcl-2 and Bcl-xl, abrogating their protective functions. This Bax translocation is independent of caspase activation, and is the first step in the apoptotic cascade (Tsuruta et al., 2004; Polster and Fiskum, 2004). Through interactions with the mitochondrial permeability transition pore complex, Bax-induced mitochondrial dysfunction leads to the release of cytochrome-C, increased generation of ROS, activation of caspases, and initiation of the execution phase of apoptosis (Marzo et al., 1998; Gross et al., 1999; Gao and Duo, 2000). Thus, Bax translocation is a critical upstream event in the apoptosis cascade. The observations in the present study of increased cytosolic and mitochondrial Bax protein following ethanol exposure are consistent with several prior studies, in which Bax mRNA and protein expression were shown to be elevated in response to ethanol both in cultured neurons, and in vivo in a number of CNS regions (e.g., Moore et al., 1999; Olney et al., 2001; Inoue et al., 2002; Heaton et al., 2003a; b; Ge et al., 2004; Schindler et al., 2004; Nowoslawski et al., 2005; Siler-Marsiglio et al., 2005a; Lee et al., 2008). Such upregulation has also previously been found to correlate with periods of heightened ethanol vulnerability, as in the present study (Moore et al., 1999; Heaton et al., 2003a,b). The importance of Bax to ethanol-mediated apoptosis in the CNS was most strikingly demonstrated in Bax gene-deleted animals, in which ethanol-induced neurodegeneration is greatly reduced or even eliminated in certain CNS regions (Young et al., 2003; Heaton et al., 2006).

3.2. The Role of JNK in Developmental Ethanol Neurotoxicity

The c-jun N-terminal kinase is a mitogen-activated protein (MAP) kinase which is activated when cells are exposed to environmental stresses such as neurotrophic factor withdrawal, elevation of reactive oxygen species, or neurotoxins such as ethanol (de la Monte, 2000; Tsuruta et al., 2004). As noted above, there are ten JNK isoforms, generated by alternative splicing of transcripts of the JNK 1, 2 and 3 genes, and are expressed as 46kDa or 55kDa proteins, which appear to be functionally redundant (Ip and Davis, 1998; Tsuruta et al., 2004; Pimienta and Pascual, 2007). When activated, one function of JNK is to phosphorylate the protective 14-3-3 cytosolic sequestering proteins, thus reducing the Bax:14-3-3 and Bad:14-3-3 binding affinities, allowing these apoptosis effectors to be liberated and travel to the mitochondrial membrane where they participate in the disruption of the mitochondrial membrane potential and interfere with the functions of protective proteins such as Bcl-2 and Bcl-xl (Siler-Marsiglio et al., 2005). Thus, JNK activation is a critical facilitator of Bax and Bad mitochondrial translocation; inhibition of this kinase prevents these movements, and suppresses cytochrome-c release and subsequent caspase activation (Gao et al., 2005; Sunayama et al., 2005; Papadakis et al., 2006). In the present study, basal

levels of JNK were greater at P4, the age of heightened ethanol sensitivity in cerebellum, and exposure at this age to ethanol elicited a striking activation of this kinase compared to exposure at the later, ethanol resistant age (P7). Similar ethanol-mediated enhancement of this pro-apoptotic kinase has previously been demonstrated in a number of regions and cell types, including neonatal rat striatum, cortex, and in neuroblastoma, and PNET neural crest tumor cells (de la Monte et al., 2000; McAlhany et al., 2000; Heaton et al., 2003a; b; Han et al., 2006). Ethanol-induced elevation of JNK activation has also been observed in non-neuronal cells (e.g., hepatocytes; Nishitani and Matsumoto, 2006; Nishitani et al., 2008). The importance of JNK activation was explored in the present study using cerebellar granule cells, in which it was possible to inhibit this activation. This portion of the study revealed that such inhibition mitigated, but did not entirely prevent, ethanol-mediated cell death within these neuronal preparations, except at the lowest ethanol concentrations (100mg/dl). Previous studies have also found chemical inhibition of JNK only partially effective in protecting cells from toxic conditions (Gao et al., 2005). This is likely attributable to additional pathways activated by the cellular stressors, other than those involving JNK activation (Gao et al, 2005).

3.2. A Role for 14-3-3 Proteins in Ethanol Neurotoxicity

The 14-3-3 proteins, named for their electrophoretic elution and migration patterns, are highly conserved dimeric eukaryotic regulatory molecules which play important roles in binding and modulating a variety of signaling proteins (Moore and Perez, 1967; Masters and Fu, 2001). These specific phosphoserine-binding proteins exist in seven isoforms in mammals, and are abundant in brain, making up an estimated 1% of the total soluble protein (Muslin et al., 1996; Ferl, 1996; Liu et al., 2010). This 14-3-3 binding serves a protective function; increases or decreases in 14-3-3 availability can shift the balance between cellular survival or cell death (Sunayama et al., 2005). More than seventy binding partners have been identified for the 14-3-3 proteins (Masters and Fu, 2001). Among these, and of particular importance to the apoptotic program, are the apoptosis agonists Bad and Bax (Watanabe et al., 1994; Nomura et al., 2003; Tsuruta et al 2004). Bax has been demonstrated to complex with at least five of the ten known 14-3-3 isoforms, i.e., sigma, eta, epsilon, zeta and theta (Nomura et al., 2003; Tsuruta et al., 2004; Gao et al., 2005). As noted above, the 14-3-3 proteins are major targets of JNK, with JNK serine phosphorylation of 14-3-3, elicited by cellular stresses, playing a major role in facilitating apoptotic activities of Bax and Bad (Tsuruta et al., 2004; Gao et al., 2005). The present study suggests that a key upstream event in ethanol neurotoxicity in developing nervous system may be the decline of intact Bax: 14-3-3 dimers seen following ethanol exposure. This decrease was found only at the age representing the peak period of ethanol sensitivity within developing cerebellum, however, with no significant alterations found at the later, ethanol-resistant age. This liberation of Bax from its cytosolic anchor, resulting from JNK phosphorylation of the cytosolic anchoring proteins, is undoubtedly facilitated by the massive increase in available pJNK following ethanol exposure at this early age, as well as by higher basal levels of JNK at the younger age. At the older age, it can also be speculated that the increased presence of the 14-3-3 proteins as a function of ethanol treatment has a protective effect, providing enhanced opportunities for Bax and Bad sequestration. Such upregulation of these anchoring proteins is typically seen in more mature neurons in response to insult or injury, and such enhanced expression has been specifically reported for the zeta and theta Bax-binding isoforms (Namikawa et al., 1998). Complexes of 14-3-3 with apoptosis-related proteins have previously been found to be influenced by ethanol treatment, findings consistent with those of the present study. Bad:14-3-3 dimers, for example, are decreased in P4 cerebellum following ethanol treatment, while such associations increase following exposure on P7 (Siler-Marsiglio et al., 2006). Similar ethanol effects on Bad:14-3-3 were subsequently found in cortical tissue at the age of peak ethanol sensitivity within that region (P7; Han et

al., 2008). In this later study, acute ethanol treatment was also demonstrated to enhance interactions between pJNK and 14-3-3, with such interactions resulting in 14-3-3 phosphorylation, followed by Bad release.

3.4 Conclusions

This study has defined upstream molecular processes which are triggered by ethanol exposure during developmental periods, and has further shown that these processes are differentially affected at ages of maximal ethanol sensitivity, compared to later ages in which the neonatal cerebellum is able to withstand ethanol insult. The following sequence of events appears to represent critical mechanisms underlying early ethanol vulnerability within this region at the age of peak sensitivity: Basal levels of JNK are high at this age, and ethanol exposure results in enhancement of JNK activation; ethanol exposure at this age also produces elevations of Bax protein; availability of the 14-3-3 proteins *does not* change as a result of ethanol insult; the enhanced activated JNK is available to phosphorylate 14-3-3, reducing the Bax:14-3-3 binding affinity, resulting in Bax release; the Bax so liberated from its cytosolic anchor travels to the mitochondrial membrane. At the later, ethanol resistant age, no alterations in JNK, Bax:14-3-3 complexes, or Bax protein are seen following ethanol exposure, except a small decrease in mitochondrial Bax; but ethanol treatment at this age results in elevations of the protective 14-3-3 proteins. Thus, changes at the vulnerable age are predictive of an apoptotic outcome, while the later changes are either neutral or protective, increasing the likelihood of cell survival. These molecular processes may represent points which can be targeted in future studies concerned with designing possible therapeutic strategies for minimizing devastative effects of developmental ethanol exposure.

4. Experimental Procedures

4.1. Ethanol Vapor Inhalation Exposure Paradigm

All procedures used in this study were carried out in compliance with the rules and regulations for the experimental use and care of laboratory animals at the University of Florida, and were approved by the Institutional Animal Care and Use Committee. For the ethanol inhalation treatment, neonatal Long Evans male and female rats (Charles River Co., Portage MI), were acutely exposed to ethanol via the vapor inhalation procedure, as previously described (e.g., Heaton et al. 2000). Briefly, litters were culled to 10 male and female pups, and at P4 or P7, pups were placed, in their home cage along with the nursing dam, in an inhalation apparatus. The Plexiglas inhalation chamber was fitted with intake and exhaust hoses, and air flow was provided by a pump which delivered approximately 0.8–1.0 liter/minute. For the ethanol groups, air flowed into an Erlenmeyer vacuum flask containing 95% ethanol. The air passed through an air stone submerged in the ethanol, and the ethanol-laden vapor was carried into the chamber. The exhaust hose led ethanol vapor into a fume hood. For control litters, the air was pumped into the chamber without exposure to ethanol. Both ethanol and control exposure periods were 2 hours, 45 minutes each day. This acute exposure protocol mimics a “binge” pattern of ethanol consumption, which is a common consumption pattern in women who drink during pregnancy (Stephens, 1985).

Two hours after the completion of exposure to ethanol or control conditions, pups were sacrificed via rapid decapitation. This timepoint was selected because it has previously been shown that ethanol-mediated changes in molecular events in the developing CNS begin shortly after exposure, and alterations in apoptosis-related molecules have been found to occur as early as one hour post-treatment (Ge et al., 2004). Following sacrifice, the brains were removed, and the cerebellum was taken for protein analyses. Blood ethanol concentrations (BECs) were determined 2 hours following completion of the inhalation exposure, using trunk blood from animals exposed in parallel to those used for the various

assays, via a kit from Diagnostic Chemical Limited, USA (Oxford, CT). For all molecular analyses, a minimum of six P4 litters and six P7 litters were exposed to control conditions and an equivalent number of litters were exposed to ethanol conditions. For Western blot analyses, cerebellae from three to five animals from each exposed litter (control and ethanol) were electrophoresed on gels for each protein tested. At least 4–5 blots were run for each experimental condition. For ELISAs, cerebellae from three to four animals from each exposed litter (control and ethanol) were pooled, and placed in microwells in triplicate; N=12–20 animals were used for each plate with one condition/plate. Three-four replicate plates were run/protein. Since no gender-related differences in the effects of ethanol at this developmental stage in the CNS of rats have been found (Andrews et al., 1999; Bonthius and West, 1991), tissues from male and female pups were pooled.

4.2. ELISA Analyses of Basal Levels of Bax, JNK and 14-3-3 Proteins

The ELISA assessments of basal levels of cerebellar JNK, 14-3-3 and Bax proteins were performed in control P4 and P7 animals to determine whether these proteins were developmentally regulated with the age span under investigation. For the JNK analyses, a kit from Calbiochem (Gibbstown, NJ) was used. This assay detected levels of JNK1 and JNK2 proteins independent of their phosphorylation status. Cerebellar tissue samples were weighed and a 5% w/v extract was prepared using a lysis buffer, according to kit instructions. The Calbiochem kit contains 8 well strips coated with a monoclonal antibody specific for JNK1/2. Experimental samples (diluted 1:6 with Standard Diluent Buffer), JNK1/2 standards (range of 0 – 20 ng/ml), and controls were added to the coated well strips. The wells were covered with plate sealer and incubated for 2 hours at room temperature (RT). The solution was decanted from the well strips, washed 4 times (4X), rabbit anti-JNK1/2 (detection antibody) added, and wells covered and incubated for 1 hour at RT. After incubation, the solution was decanted, rinsed 4X and anti-rabbit IgG-HRP Working Solution added. The wells were covered and incubated for 30 minutes at RT. After incubation, solution was decanted from the wells, they were rinsed 4X and Soluble Substrate added. The wells were sealed with plate sealer and incubated in the dark for 30 minutes. The liquid in the wells turned blue and the absorbance was read on a microplate reader at 450nm and the values were normalized per gram of tissue assayed. At least 6–8 ethanol-exposed and control animals were used for these analyses at each age.

For the 14-3-3 and Bax assessments, the Express ELISA Kit from GenScript (Piscataway, NJ) was used with a primary antibody from Chemicon which recognized all 14-3-3 isoforms, and a primary Bax antibody from Cell Signaling (Danvers, MA). Microplates were coated with the 14-3-3 primary antibodies (diluted 1:200 with coating buffer in each instance) and incubated for 5 minutes at RT. Coating solution was removed and plates blocked with Pretreat Solution for 5 minutes at RT. The pretreat solution was then removed, the plates washed 3X with wash solution, samples added, and the plates incubated for 30 minutes at RT. The Pretreat solution was then removed, the plates washed twice, ELISA solution (from the kit) added, and plates incubated for 20 minutes at RT. Following incubation, the plates were washed 5X and One-Solution (color development with tetramethylbenzidine) was added for a 15-minute incubation. The enzyme reaction was stopped by adding Stop Solution (the blue reaction mixture turns yellow) and absorbance was read and the values were normalized as above.

4.3. Subcellular Fractionation

For the analyses of subcellular Bax localization, cerebellar tissue was fractionated, in order to separate cytosolic and mitochondrial contents. For this procedure, P4 or P7 cerebellae were harvested 2 hours following termination of exposure to ethanol or control conditions. The tissues were homogenized and isolated into fractions using the Mitochondrial/Cytosol

Fractionation Kit and protocol provided by BioVision Inc. (Mountain View, CA). Tissues were suspended in 700 ml of 1X Cytosol Extraction Buffer containing protease inhibitor cocktail and dithiothreitol (DTT). Tissue samples were incubated for 30 minutes on ice and homogenized by 80–100 passes in an ice-cold dounce tissue grinder. The homogenates were transferred to new tubes and centrifuged at 2000 rpm for 10 minutes at 4°C to remove nuclei, membranes, and whole cells. A 550µl portion of the supernatant was transferred to a new tube, with care being taken to leave the pellet undisturbed. The supernatant was centrifuged at 14,000 rpm for 30 minutes at 4°C, then retained as the cytosolic fraction. The remaining supernatant on the pellet was removed, and the pellet was rinsed by adding 300µl of 1X PBS. This mixture was then vortexed and centrifuged at 14,000 rpm for 30 minutes at 4°C. Supernatant was removed from the vortexed pellet, and 195µl of Mitochondrial Extraction Buffer added. The suspension was sonicated with an ultrasonic cell disruptor (from Misonix Inc., Farmingdale, NY) to release the mitochondrial contents, and this constituted the mitochondrial fraction. Protein content of cytosolic and mitochondrial fractions was quantified using bicinchoninic acid (BCA) protein assays (Pierce, Rockford, IL). Protein concentrations of fractions exposed to each condition were normalized for molecular analyses.

The quality of the separation of the fractions using this procedure was determined by Western blot of mitochondrially-specific cytochrome oxidase subunit Vb (Cox IV), using a monoclonal mouse antibody (Molecular Probes, Carlsbad, CA). No COX IV was detected in the cytosolic fraction, while abundant levels were found in the mitochondrial fractions, as previously reported using this fractionation procedure (Heaton et al., 2011).

4.4. Western Blot Analyses of Ethanol Effects on JNK, 14-3-3 and Bax Proteins

Effects of ethanol on expression of the phosphorylated JNK kinase, the 14-3-3 proteins, and cytosolic and mitochondrially translocated Bax were determined using Western blots assessed 2 hours post-ethanol treatment. Whole tissue samples were homogenized with an ultrasonic cell disruptor in lysis buffer (137mM NaCl, 20mM Tris, 1% NP40, 10% glycerol, 1 mM PMSF, 10µg/ml aprotinin, 1µg/ml leupeptin, 0.5mM sodium vanadate). The buffer contained a cocktail of protease inhibitors, containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E64, bestatin, leupeptin, and aprotinin (Sigma; St. Louis, MO). The extracts were then centrifuged for 7 minutes at 10,000g. The protein content of the supernatant was analyzed via the BCA protein assay kit (Pierce, Rockford, IL). Samples (whole tissue as well as cytosol/mitochondrial fractions) were diluted with Laemmli sample buffer (BioRad, Hercules, CA). The proteins were heated for 5 minutes at 100°C, then cooled for 5 minutes. Proteins were resolved via polyacrylamide gel using pre-cast, 8–16% SDS-polyacrylamide gradient gels, and utilizing a BioRad electrophoresis unit, with 25µg protein loaded per lane. Proteins were transferred to nitrocellulose membranes (0.45µm pore size, from BioRad), in a BioRad wet transfer unit. After transfer was completed, the gels were stained with Coomassie Blue to verify successful protein separation, and the membranes were stained with Ponceau Red to confirm successful transfer. Membranes were blocked with tris-buffered saline (TBS) with 0.05% Tween-20, and 5% Blotto (non-fat dry milk; Santa Cruz, Santa Cruz, CA) for 1 hour at RT. Membranes were then probed overnight at 4°C with the appropriate antibodies: For detection of activated JNK, membranes were probed with antibodies to activated JNK (pJNK), using the anti-ACTIVE-JNK polyclonal antibody, which recognizes the dually phosphorylated Thr/Pro/Tyr regions within the catalytic domain of active JNK (1:1000; Promega, Madison, WI). These membranes were then blocked with BSA, since the pJNK antibody is sensitive to milk proteins. The 14-3-3 family includes seven isoforms, and at least five of these bind to Bax (sigma, eta, and epsilon, zeta and theta; Gao et al., 2005). Therefore, for these analyses, we used a pan-14-3-3 antibody which recognizes all isoforms (1:1000; Chemicon/Millipore, Temecula,

CA). For the Bax Westerns, a polyclonal antibody from Cell Signaling (1:1000) was used. For the 14-3-3 and Bax blots, membranes were blocked with TBS with Tween-20, and Blotto dried milk. Cyclophilin-40 was used as an internal control in tissues from experimental and control animals, as in our previous studies (e.g., Siler-Marsiglio et al., 2006). This protein was used to verify loading equivalence, and enabled us to determine whether observed effects are ethanol-specific rather than global. A polyclonal antibody from Calbiochem (1:1000) was used for these controls.

Following incubation with the primary antibodies, membranes were probed with an anti-rabbit HRP-conjugated secondary antibody for one hour at RT (Santa Cruz; 1:4000). Antibody specificity was verified by molecular weights of the bands and by the use of blocking peptides and positive controls, supplied by the various vendors. A biotinylated-protein ladder (BioRad) was fractionated on each gel and HRP-conjugated anti-biotin (diluted 1:5000; BioRad) was added to membranes to indicate molecular weights (MWs) of the detected protein bands on film.

Signals were detected by enhanced chemiluminescence using the Western blot detection kit RPN 2106 (Amersham Pharmacia Biotech; Piscataway, NJ). Following incubation in the detection reagent (usually for 1 minute), the membranes were placed on Kodak X-OMAT film in a film cassette, and the film was developed by standard photographic procedures. Quantification of signal strength was made by scanning the films with an UMAX Ultra Scanner, and densitometric analyses were made using the Scion Image computer program (Scion corp., Frederick, MD). At least 3 replicate blots per protein were performed, with at least 6 animals per gender pooled for each sample. A minimum of 4–5 gels/condition were used for analyses.

4.5. ELISA Assessment of Bax:14-3-3 Interactions

Changes in native protein-protein interactions between Bax and 14-3-3 following ethanol exposure were determined using an ELISA-based approach (Siler-Marsiglio et al., 2005). Our approach utilizes capture antibodies which recognize native protein for the detection of endogenous protein-protein interactions. Specifically, mouse monoclonal pan-14-3-3 antibody (Chemicon; diluted 1:500 in pH 9.6 bicarbonate buffer [capture antibody]) was bound to 96-well microplates overnight at 4°C. Plates were blocked for 1 hour at RT with 1% Blotto non-fat dry milk in pH 7.2 PBS and then washed 3X using PBS. Each cerebellum exposed to ethanol or control conditions was suspended in 400ml of PBS with 1% protease inhibitor 2 hours following completion of the exposure procedure. The suspension was sonicated to lyse the cells to release the endogenous Bax complexes. Protein content was determined using the BCA assay. Tissue extracts were prepared from ethanol and control animals 2 hrs following treatment. Equal amounts of protein (125µg/well) were placed in the 14-3-3 antibody-bound 96-well microplates at a final volume of 100µl/well. Plates were incubated overnight at 4°C and then washed 3X with PBS. Primary antibody was added to each plate to detect 14-3-3-bound Bax. Rabbit polyclonal anti-Bax (Cell Signaling; 1:200) was added to anti-14-3-3 plates to detect Bax:14-3-3 complexes. Following an overnight incubation at 4°C, plates were washed 3X with PBS. Anti-mouse secondary antibody conjugated to alkaline phosphatase (diluted 1:250, Sigma) was added to the anti-14-3-3 plates. Plates were incubated overnight at 4°C and washed 3X with TBS to avoid inhibition of alkaline phosphatase by inorganic phosphate. All buffers used, including the dilution and wash buffer, were detergent-free with 1% protease inhibitor cocktail. To colorimetrically detect signal from Bax:14-3-3, p-nitrophenyl phosphate (pNPP; 2mg/ml), an alkaline phosphatase substrate, in pH 10.4 glycine buffer with 1 mM ZnCl₂ was added. Absorbance was read at 415nm on a BioRad microplate reader (Hercules, CA). Absorbance readings from background wells (obtained from wells excluding primary antibody) were subtracted from experimental wells to determine specific binding.

This ELISA-based approach has the advantage of minimizing the disruption of native protein-protein interactions. It is important that our method circumvents the need for detergents: Protein interactions with Bax, for example, are induced or inhibited by detergents commonly used in immunoprecipitation procedures including Nonidet P-40, Triton X-100 and CHAPS (Hsu and Youle, 1997; 1998). Cross-linking agents, which can also adjust binding characteristics of proteins, are also avoided by our approach.

4.6. Cerebellar Granule Cell Cultures

Cerebellar granule cell cultures were used to further assess the role of the JNK kinase in ethanol neurotoxicity. As noted above, this pro-apoptotic kinase, once activated, has the potential to phosphorylate the 14-3-3 proteins, thus weakening the Bax:14-3-3 binding, and liberating Bax to travel to the mitochondrial membrane, where it contributes to the disruption of the membrane potential, triggering the apoptosis cascade. For these preparations, P8 pups were sacrificed by rapid decapitation. The cerebellum was removed, minced, and placed in Hank's balanced salt solution (HBSS) containing trypsin and DNase (Sigma). The tissue was incubated at 37°C for 15–20 minutes, washed with Dulbecco's modified Eagle's medium (DMEM; Cellgro, from Fisher Scientific; Pittsburgh, PA) with 15% fetal bovine serum (Invitrogen; Camarillo, CA), penicillin/streptomycin (Fisher), fungizone (Invitrogen), and trypsin inhibitor (Sigma), then triturated using a flame-narrowed borosilicate pipette to yield a homogeneous suspension. The cells were centrifuged, the supernatant removed, and fresh medium added to the pellet. After gentle aspiration, the cell suspension was filtered through a Nitrex filter (Sefar America, Kansas City, MO) to enrich the preparations for the small granule cells. For the assessments of JNK inhibition and neuronal survival via the MTT assay, aliquots of the cell suspension were pipetted into 96-well plates coated with poly-L-lysine (0.05mg/ml; Sigma). All cultures were established for 24 hours in a tissue culture incubator at 37 C, with 5% CO₂-95% air, in the serum-containing medium. Cell densities in these preparations average 35,000–39,000 neurons/cm², as determined by a hemocytometer. We have found cells prepared in this manner to be ~95% neuronal, as determined by immunochemical detection of glial fibrillary acidic protein (GFAP) and type III β -tubulin.

4.7. Application of Experimental Conditions

After the initial 24-hour plating period, the serum-containing medium was replaced with serum-free, modified N2 medium consisting of DMEM/F12 medium at a 3:1 ratio, 5 mM KCl, and 1% N2 supplement, containing transferrin, insulin, progesterone, putrescine, and selenite (Invitrogen). Addition of Penicillin/Streptomycin and Fungizone was equivalent to that of the serum-containing medium. Use of the serum-free defined medium eliminates contamination from unknown serum components, and discourages proliferation of any non-neuronal cells which survive the filtration procedure (Muller et al., 1997). The experimental conditions then applied included three ethanol concentrations, 100, 250 and 400mg/dl, which represent moderate-to-relatively high levels, but are well within the range seen both in awake humans following binge consumption, and in animal studies of ethanol toxicity (e.g., Young et al., 2003). Ethanol was coupled with the JNK inhibitor 1,9-pyrazoloanthrone (SP600125; Sigma), a selective, small molecule, cell-permeable, reversible anthrapyrazolone inhibitor of *c*-Jun N-terminal kinase (JNK) (Bennett et al., 2001). The inhibitor was applied at concentrations of 200nM, 10 μ M, 20 μ M, and 30 μ M, which represents the range of concentrations reported in the literature (e.g., Bennett et al., 2001; Lee and Shukla, 2005). This range was used to define the effective concentration in our preparations. The following experimental conditions were applied, for an interval of 24 hours: (1) Control, in which the N2 medium was replaced without further supplementation; (2) Ethanol, in which 100, 250 or 400mg/dl ethanol was added to the N2 medium; (3) JNK inhibitor at one of the concentrations noted above; and (4) JNK inhibitor + ethanol, with the

various combinations of conditions, as above. The culture dishes were sealed with sterile breathable plate sealer, to minimize ethanol evaporation. Ethanol concentrations were confirmed by the Diagnostic Chemical Limited microenzymatic assay, and evaporation during the short exposure time was minimal.

4.8. MTT Neuronal Survival Assay

In order to assess neuronal viability, the MTT assay was used. For this procedure, the formazan product is produced as a result of the cleavage of the tetrazolium salt MTT by the inner mitochondrial membrane enzyme succinate-dehydrogenase. Only living cells with functional mitochondria can make the conversion, and therefore the amount of blue formazan production is directly proportional to the number of viable cells present (Mosmann, 1983). This MTT reduction has previously been shown to produce highly accurate measurements of neuronal number in non-proliferative preparations such as cerebellar granule cells (Ankarcrona et al., 1995). Such measures are directly proportional to quantification of these cells by light microscopic observations (Manthorpe et al., 1986), and correlate with additional measures of cell viability such as Annexin V, and Propidium Iodide staining (Siler-Marsiglio et al., 2003). This assay was initiated 24 hours following application of experimental conditions. The protocol for the MTT survival assay was as outlined in detail previously (Mitchell et al., 1999). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was prepared in phenol red-free Eagle's Minimum Essential Medium. The granule cell cultures were incubated in the MTT solution for 5 hours at 37°C, resulting in production of the formazan product as a result of the mitochondrial cleavage. Following incubation, the plates were centrifuged, and the untransformed MTT solution was removed. Propanol was added to each well and the plates were shaken to uniformly solubilize the blue formazan. The optical density (OD) of each well was measured using an automated plate reader (BioRad) with a test wavelength of 550 nm. Only one ethanol concentration was used on any ethanol-containing plate. Thus, each plate contained multiple wells of a given experimental condition and multiple control wells, and mean OD readings were compiled for columns of 4–12 wells (fewer wells are used for the ethanol-supplemented cultures to prevent diffusion to control wells (Mitchell et al., 1998). This procedure was replicated a minimum of 8 times, on 3–4 plates/condition.

4.9. Statistical Analyses

Statistical comparisons of basal levels of Bax, JNK and 14-3-3 proteins in the P4 and P7 cerebellum were made via the Student's *t*-test. The effects of ethanol on P4 and P7 Bax, JNK and 14-3-3 protein expression, on Bax:14-3-3 protein-protein interactions, and on survival of cerebellar granule cells following experimental treatments were made via the two-way analysis of variance (ANOVA) using StatView software on a Macintosh computer. Posthoc comparisons were made with the Fisher's Protected Least Significant Difference (PLSD) test, where appropriate. All data were obtained from at least three independent experiments.

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Highlights

- Ethanol-induced apoptosis in neonatal CNS involves Bax mitochondrial translocation.
- Ethanol-induced apoptosis in neonatal CNS involves enhanced JNK activation.
- Ethanol-induced apoptosis in neonatal CNS involves decreased Bax:14-3-3 dimers.
- JNK inhibition blunts ethanol-induced apoptosis in cerebellar granule cells.
- Differential ethanol sensitivity can be related to events affecting Bax activation.

Figure 1. Basal levels of the c-jun N-terminal kinase (JNK) in postnatal day 4 (P4) rat cerebellum exceed those found at postnatal day 7 (P7). Basal levels JNK were measured via ELISA assay in P4 and P7 neonatal rat cerebellum. Error bars represent standard error of the mean (SEM). a=Significantly greater than P7, $p=0.0013$.

Figure 2.

Activated JNK in neonatal rat cerebellum is enhanced by ethanol treatment at P4 but not at P7. (A) Activated (phosphorylated) c-jun N-terminal kinase (pJNK) was measured by quantification of optical density (OD) levels of Western blots from P4 and P7 neonatal rat cerebellum two hours following exposure to ethanol via vapor inhalation (Etoh), or to control conditions (Con). Error bars represent standard error of the mean (SEM). a=Significantly greater than P4 controls, $p=0.0047$. (B) Representative Western blots of pJNK protein from cerebellum of control and ethanol-treated P4 and P7 neonates. The arrows point to the 46kDa JNK band, which was predominant in our analyses. The two blots/condition are from different animals, from different litters. (C) Representative Western blots of cerebellar cyclophilin-40 protein, used as a loading control and to assess the generality of the ethanol-mediated alterations, are shown, from P4 and P7 neonates from the two experimental conditions. The arrows point to the 40kDa cyclophilin-40 band.

Figure 3.

The 14-3-3 proteins in neonatal rat cerebellum are enhanced by ethanol treatment at P7 but not at P4. (A) The 14-3-3 proteins, using a pan-14-3-3 antibody which recognizes all isoforms, were measured by quantification of optical density (OD) levels of Western blots from P4 and P7 neonatal rat cerebellum following exposure to ethanol via vapor inhalation (EtOH), or to control conditions (Con). Error bars represent standard error of the mean (SEM). a=Significantly greater than P7 controls, $p=0.0145$. (B) Representative Western blots of 14-3-3 protein from cerebellum of control and ethanol-treated P4 and P7 neonates. The two blots/condition are from different animals, from different litters.

Figure 4.

Bax protein in cytosolic fractions of neonatal rat cerebellum is increased following ethanol treatment at P4 but not at P7. (A) Cytosolic Bax was measured by quantification of optical density (OD) levels of Western blots from isolated cytosolic fractions derived from P4 and P7 neonatal rat cerebellum following exposure to ethanol via vapor inhalation (Etoh), or to control conditions (Con). Error bars represent standard error of the mean (SEM).

a=Significantly greater than P4 controls, $p=0.0005$. (B) Representative Western blots of cytosolic Bax protein from cerebellum of control and ethanol-treated P4 and P7 neonates. The two blots/condition are from different animals, from different litters.

Figure 5.

Bax protein in mitochondrial fractions of neonatal rat cerebellum is increased following ethanol treatment at P4 but not at P7. (A) Bax mitochondrial translocation was measured by quantification of optical density (OD) levels of Western blots from isolated mitochondrial fractions derived from P4 and P7 neonatal rat cerebellum following exposure to ethanol via vapor inhalation (Etoh), or to control conditions (Con). Error bars represent standard error of the mean (SEM). a=Significantly greater than P4 controls, $p < 0.0001$; b=significantly less than P7 controls, $p = 0.016$. (B) Representative Western blots of mitochondrial Bax protein from cerebellum of control and ethanol-treated P4 and P7 neonates. The two blots/condition are from different animals, from different litters.

Figure 6.

Bax:14-3-3 heterodimers are decreased in neonatal rat cerebellum following ethanol treatment at P4 but not at P7. Bax:14-3-3 heterodimers were measured by ELISA in tissue derived from P4 and P7 neonatal rat cerebellum following exposure to ethanol via vapor inhalation (Etoh), or to control conditions (Con). Error bars represent standard error of the mean (SEM). a=Significantly less than P4 controls, $p < 0.0001$.

Figure 7.

Four concentrations of JNK inhibitor are equally effective in ameliorating ethanol neurotoxicity in neonatal cerebellar granule cells. Postnatal day 8 cerebellar granule cells were cultured in control medium (Con), in medium supplemented with 400mg/dl ethanol (Et400), and in medium supplemented with Et400 plus the JNK inhibitor SP600125 at concentrations of 200 M, 10µM, 20µM and 30µM. Neuronal survival was measured by the MTT assay. Error bars represent standard error of the mean (SEM). a=Significantly less than controls, $p < 0.0001$; b=significantly greater than ethanol alone, $p < 0.0005$.

Figure 8.

Inhibition of JNK enhances survival of cerebellar granule cells cultured with ethanol. Postnatal day 8 cerebellar granule cells were cultured in control medium (Con), in medium supplemented with JNK inhibitor SP600125 (Ji), in medium supplemented with 100, 250 or 400mg/dl ethanol (Et100, Et250, Et400), and in medium with ethanol plus the JNK inhibitor (Et100+Ji; Et250+Ji; Et400+Ji). Since no differences were detected between the four inhibitor concentrations assessed (200nM, 10 μ M, 20 μ M and 30 μ M), the data were combined for the analyses depicted in this figure. Neuronal survival was measured by the MTT assay. Error bars represent standard error of the mean (SEM). a=Significantly less than controls, $p < 0.0001$; b=significantly greater than ethanol alone, $p < 0.0005$.