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Mechanisms of Ethanol-induced Death of Cerebellar Granule Cells

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Abstract

Maternal ethanol exposure during pregnancy may cause fetal alcohol spectrum disorders (FASD). FASD is the leading cause of mental retardation. The most deleterious effect of fetal alcohol exposure is inducing neuroapoptosis in the developing brain. Ethanol-induced loss of neurons in the central nervous system (CNS) underlies many of the behavioral deficits observed in FASD. The cerebellum is one of the brain areas that is most susceptible to ethanol during development. Ethanol exposure causes a loss of both cerebellar Purkinje cells and granule cells. This review focuses on the toxic effect of ethanol on cerebellar granule cells (CGC) and the underlying mechanisms. Both *in vitro* and *in vivo* studies indicate that ethanol induces apoptotic death of CGC. The vulnerability of CGC to ethanol-induced death diminishes over time as neurons mature. Several mechanisms for ethanol-induced apoptosis of CGC have been suggested. These include inhibition of NMDA receptors, interference with signaling by neurotrophic factors, induction of oxidative stress, modulation of retinoid acid signaling, disturbance of potassium channel currents, thiamine deficiency, and disruption of translational regulation. Cultures of CGC provide an excellent system to investigate cellular/molecular mechanisms of ethanol-induced neurodegeneration and to evaluate interventional strategies. This review will also discuss the approaches leading to neuroprotection against ethanol-induced neuroapoptosis.

Keywords

Apoptosis; cell signaling; development; fetal alcohol exposure; neurodegeneration; neuroprotection

Introduction

Fetal Alcohol Spectrum Disorders (FASD) are a range of permanent birth defects caused by maternal alcohol consumption during pregnancy. FASD is characterized by a spectrum of structural anomalies; one of the most severe consequences of prenatal alcohol exposure is the disruption of brain development, resulting in structural brain anomalies (1-3) and neurocognitive and neurobehavioral impairment (4-6). FASD currently represents the leading cause of mental retardation (7-9). The incidence of FASD is estimated as high as 1 in 100 births (10), and the cost of dealing with the consequences of prenatal alcohol exposure is estimated at \$4 billion per year in the United States (11).

One consistent finding regarding ethanol-induced brain abnormalities is microencephaly and the permanent loss of neurons. The cerebellum is the motor coordination center of the

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central nervous system (CNS) and is also involved in cognitive processing and sensory discrimination. The developing cerebellum is particularly sensitive to ethanol exposure. Structural magnetic resonance imaging (MRI) studies show that children and adolescents with a history of prenatal ethanol exposure display a reduction in cerebellar volume and a decrease in the size of the vermis (12-17). Children with FASD show many symptoms associated specifically with cerebellar damage (17,18). Experimental studies using animal models have confirmed that developmental exposure to alcohol reduces the size of the cerebellum and causes the loss of cerebellar Purkinje cells and granule cells, two major neuronal populations in the cerebellum (19-21). The majority of animal studies document the toxic effect of ethanol on Purkinje cells and less attention has been given to the damage in cerebellar granule cells (CGC) *in vivo*. In contrast to the relatively small amount of *in vivo* studies that specifically investigate the effect of ethanol on CGC, there is abundant research studying the interaction between ethanol and CGC in a cell culture system. This is because a large amount of CGC can be generated from postnatal rodent cerebellum, and cultured CGC undergo a maturing process, exhibiting the neuronal characteristics observed *in vivo*. In addition, cultured CGC offer a well controlled system which minimizes many confounding factors in animal models.

1. Ethanol-induced death of CGC

In adult mammals, the cortex of the cerebellum contains three layers. The most superficial one is the molecular layer (ML). The deepest layer contains the granule cells and is therefore called the granular layer (GL). At the interface between these two layers, a thin middle zone exists in which the bodies of the Purkinje cells (PCs) are aligned into a single row; this layer is the Purkinje cell layer (PCL). Different cell types in the cerebellum develop at different times. The Purkinje cells in rats are formed during days 14–16 of gestation; at birth they form a layer that is several cells thick which then transforms into a single cell layer by postnatal days (PD) 3–4. The external germinal layer (EGL) is formed around the same time that PCs are generated. During the first postnatal week, CGC precursors (CGCP) in the EGL proliferate massively and postmitotic cells reach the deep part of the EGL and begin to differentiate, as evidenced by the extension of parallel fibers (axonal processes). After neurite extension commences, the cell body is polarized and attaches to the fibers of the Bergmann glia, which serve as a substrate for radial migration through the ML. CGC migrate to their destination, the internal granular layer (IGL) where they extend dendrites and continue the maturing process. As the migration process completes, the EGL disappears between postnatal weeks 2-3 (22-24).

Researchers have designed rodent models of developmental ethanol exposure. One of the effective models administers ethanol to neonatal rodents. The developmental stage of the neonatal rodent brain, which is rapid during synaptogenesis, is equivalent to the last trimester of the human fetus (25,26). Several routes of ethanol administration have been developed. These include: (1) artificial rearing with surgical implantation of a feeding tube; (2) acute intragastric intubation; (3) vapor inhalation; and (4) subcutaneous injection. Similar to FASD patients, the rodent models show brain abnormalities, such as microencephaly and neuron loss (27-29), delayed myelination (30), and behavioral abnormalities (31,32).

The rat cerebellum is particularly vulnerable to ethanol during the first 10 days after birth. Ethanol exposure during this time period causes depletion of both Purkinje and granule cells (19-21,33). This is a period corresponding with Purkinje cell dendritic outgrowth and synaptogenesis. Purkinje cells are depleted after exposure to doses of ethanol that raise peak blood alcohol concentrations (BACs) above 180 mg/dl (binge-like exposure) during PD4-9 (34). Depletion has a regional difference, with the lobules I-V and VIII-X generally showing the most depletion. Depletion appears to be independent of the ways that ethanol is delivered

to pups. Compared to Purkinje cells, CGC may have greater sensitivity to ethanol; while Purkinje cells are reduced by approximately 20% compared to controls when BACs are 300 mg/dl or greater, CGC are reduced by 20% at lower BACs (less than 150 mg/dl), and show as much as 40% depletion when peak BACs exceed 400 mg/dl (35). Similar results are observed in the mouse model. In this model, ethanol is administered subcutaneously at a total dose of 5 g/kg (2.5 g/kg at time zero and 2.5 g/kg again at 2 hours)(32). This dosing regimen produces a sustained elevation of BACs above 200 mg/dl for at least 8 hours. Ethanol exposure during early postnatal days (PD2-PD9) causes neuroapoptosis of Purkinje and CGC in mice (32,36).

Although some investigators suggest that granule cells losses may occur as a delayed reaction secondary to the loss of Purkinje cells (37-39), other evidence indicates that ethanol may cause death in granule cells by a direct mechanism (36). In addition, *in vitro* studies using primary cultures of purified CGC show ethanol exposure at physiologically relevant concentrations causes CGC death, supporting the notion that ethanol can kill CGC directly (40,41).

In the rat model system of FASD, the vulnerability of cerebellar neurons to ethanol-induced death diminishes over time (21,33,42). Ethanol exposure at PD4-5 kills more cerebellar neurons than a similar exposure at PD8-9. Furthermore, restriction of ethanol to the narrow period of PD4-5 kills as many cerebellar neurons as daily exposure over the much broader period of PD4-9 (33). Siler-Marsiglio et al. (2005) also show that the cerebellum of rats at PD4 is more vulnerable to ethanol than at PD7(43). These results demonstrate a temporal window of vulnerability to ethanol exposure during development. This observation is supported by *in vitro* studies showing that CGC acquire a more mature neuronal phenotype with continued time in culture and the vulnerability of cultured CGC to ethanol-induced death diminishes over time (40).

Animal studies indicate that ethanol-induced death of CGC in the cerebellum is likely in the form of apoptosis (36,44). *In vitro* studies using CGC cultures confirm these results; at the range of 200-800 mg/dl, ethanol causes the death of freshly isolated CGC in a concentration dependent manner (45-54). The death is characteristic of apoptosis which is indicated by the increase of caspase-2, -3, -6, -8, and -9 activities, DNA fragmentation, and mitochondrial permeability (45-54).

2. Mechanisms of ethanol-induced death of CGC

Primary CGC cultures (greater than 90% purity) provide a well-defined biological system in which experimental conditions can be precisely controlled and manipulated, and the direct effects of ethanol can be evaluated. This system has been extensively used to study the mechanisms of ethanol-induced neuronal death. In this model system, CGC are generally isolated from rats or mice at the age of PD7-10 and maintained in a depolarizing condition (25 mM of KCl). CGC under this condition do not proliferate *in vitro*, therefore this is an ideal model to study ethanol-induced cell death. The overall effect of ethanol on CGC survival reflects combined outcomes of the promotion of pro-apoptotic pathways and the inhibition of neurotrophic action.

Interference with N-methyl-D-aspartate (NMDA)'s action—CGC can be maintained in culture for relatively long periods if they are grown in the presence of a depolarizing concentration (25 mM) of KCl (55). When CGC are cultured in the presence of a physiological concentration of KCl (5 mM), however, they are considered to be “immature” neurons which undergo apoptotic death (56). This death can be prevented by treating the cells with NMDA. In CGC cultures, ethanol, added simultaneously with NMDA, attenuates this protection through the inhibition of NMDA receptor function, leading to enhanced

apoptosis (45-47,52,57). It has been reported that in an adult brain, ethanol acutely inhibits NMDA receptor function, and the NMDA receptor is up-regulated following chronic ethanol exposure and withdrawal (58). Ethanol inhibition of the anti-apoptotic effect of NMDA is associated with a change in the properties of the NMDA receptor. This is indicated by decreased ligand binding, decreased expression of NMDA receptor subunit proteins, and decreased functional responses including stimulation of increases in intracellular Ca^{2+} and induction of brain-derived neurotrophic factor (BDNF) expression (57).

The neuroprotective/neurotrophic effects of NMDA on CGC may be mediated by multiple mechanisms. First, the neurotrophic effect of NMDA is thought to reflect *in vivo* innervation of developing CGC by glutamatergic afferents (57). Second, the protective effect of NMDA may be mediated by increasing intracellular Ca^{2+} (46,47). Inhibition of CGC survival by ethanol corresponds with a marked reduction in the capacity of NMDA to increase the concentration of intracellular Ca^{2+} . Third, NMDA can stimulate the production of nitric oxide (NO), which can in turn enhance the synthesis of cyclic GMP and offer neurotrophism for CGC (59). Fourth, NMDA may induce the expression of neurotrophic factors, such as BDNF, or activate intracellular neurotrophic signaling pathways, such as insulin receptor-mediated signaling (46,57). Fifth, NMDA may activate neurotrophic transcription factors, such as NF- κ B (60). Ethanol exposure blocks these neuroprotective/neurotrophic effects of NMDA, causing neurotoxicity. Other studies demonstrate that exogenous NMDA can counteract ethanol-induced apoptosis of CGC and offer neuroprotection (46,61). NMDA's protection against ethanol-induced cell death is believed to be mediated by a mechanism that involves the NO-cyclic GMP and PI3K pathways (46,59).

However, there is some evidence that does not support the involvement of NMDA receptors in ethanol neurotoxicity. For example, an NMDA receptor antagonist (MK801) does not alter rates of ethanol-induced death of CGC in culture (50). In contrast to most evidence showing that NMDA is a neuroprotective/neurotrophic agent for cultured CGC, Cebere and Liljequist (2003) demonstrate that NMDA or a NR2A-B subunit-selective NMDA receptor agonist, homoquinolinic acid (HQ), induces CGC death; ethanol antagonizes NMDA's and HQ's action and is neuroprotective for CGC (62).

Interference with signaling by neurotrophic factors—Neurotrophins and growth factors play an important role in maintaining neuron survival during brain development. Interference with neurotrophic signaling is a potential mechanism for ethanol-induced damage to the CNS (63). Insulin and insulin-like growth factor I (IGF-I) promote the survival of cultured CGC through a PI3K-dependent pathway (41,47). In a model of chronic gestational ethanol exposure, rats were exposed to ethanol throughout pregnancy. CGC were isolated from PD2 pups born from control or ethanol-fed rats. Prenatal ethanol exposure reduces the expression of insulin, IGF-II, and the IGF-I and IGF-II receptors; it also inhibits insulin and IGF-I-stimulated signaling in CGC (64,65). In addition, prenatal ethanol exposure induces oxidative stress as well as decreases cell viability and the expression of pro-apoptotic genes (p53, Fas-receptor, and Fas-ligand) in cultured CGC. The *in vivo* results confirm that prenatal ethanol exposure inhibits insulin and IGF-I-stimulated cell signaling by impairing the receptor tyrosine kinase (RTK) activities in the cerebellum (65). Similarly, *in vitro* ethanol exposure also inhibits IGF-I receptor activation in cultured CGC (66). Ethanol inhibits IGF-I-mediated neuronal survival, but does not inhibit IGF-I receptor binding or the neurotrophic action of elevated K^+ (47). Inhibition of neuronal survival by ethanol is not reversed by increasing the concentration of exogenous IGF-I. The inhibition of IGF-I-mediated protection by ethanol corresponds to a marked reduction in the phosphorylation of insulin receptor substrate 1 and IGF-I-stimulated PI3K activity (47).

BDNF is a neurotrophic factor for CGC (48). Acute exposure to ethanol causes down-regulation of mRNA for BDNF and its receptor TrkB in the cerebellum of rats at PD4, the peak period of cerebellar vulnerability to ethanol (67,68). However, Heaton et al. (1999) show that ethanol exposure at this period reduces the protein levels of nerve growth factor (NGF) protein, but not BDNF (69). *In vitro* ethanol exposure inhibits the secretion of BDNF and neurotrophin-3 (NT-3) from cultured CGC (70). Further studies demonstrate that ethanol exposure blocks BDNF signaling in cultured CGC which is evident by a diminished response to BDNF-stimulated activation of ERK and PI3K (71,72). BDNF stimulates activator protein-1 (AP-1) transactivation in cultured CGC via PI3K and JNK pathways. Ethanol inhibits BDNF-mediated activation of PI3K/Akt and JNKs, and blocks BDNF-stimulated AP-1 activation. Since ethanol does not affect either the expression or autophosphorylation of TrkB, the site of ethanol action may be downstream of TrkB (71).

Involvement of retinoic acid signaling—Retinoic acid (RA) is a physiologically active metabolite of vitamin A that is locally synthesized in the cerebellum (73). The levels of RA, its receptors and binding proteins are developmentally regulated in the cerebellum (73,74). Although RA functions as an endogenous regulator of cerebellar development, abnormal expression of RA may be teratogenic (75). The cellular effects of RA are mediated by two classes of receptors: retinoid receptors (RARs) and rexinoid receptors (RXRs). As the cerebellum expresses a high number of RXR receptors and RXR targets genes predominantly involved in apoptosis, it is possible that ethanol activates RXRs to induce apoptosis of CGC. Kumar et al. (2009) show that ethanol reduces the expression of RAR α / γ while it increases the expression of RXR α / γ in the cerebellum of PD7 rats (76). Similar results are observed in cultured CGC. The observation suggests that ethanol may promote harmful effects in CGC by targeting RA receptor-mediated signaling.

Involvement of intrinsic apoptotic signaling—Ethanol-induced death of CGC is mainly mediated by the mitochondrial apoptotic pathway (also known as intrinsic apoptotic pathway)(77-78). The Bcl-2 family of proteins is a critical component of intrinsic apoptotic pathway and comprises of both pro-apoptotic and anti-apoptotic members. The stoichiometry of pro- versus anti-apoptotic Bcl-2 family members in the cell determines whether the cell lives or dies (79). Acute exposure to ethanol up-regulates pro-apoptotic Bax, Bad, and Bcl-xs, but down-regulates anti-apoptotic Bcl2 in the cerebellum of PD4 rats (80-82).

Using a Bax deficient mouse model, Nowoslawski et al. (2005) demonstrate that ethanol produces extensive Bax-dependent caspase-3 activation and neuron apoptosis in the IGL of the developing cerebellum, which is maximal at 6 hours post-administration (78). This effect is recapitulated *in vitro* using CGC culture isolated from Bax-deficient mice. Ethanol-induced neuron death is unaffected by a deficiency in the pro-apoptotic Bcl-2 family members Bid or Bad. Heaton et al. (2006) also demonstrate that Bax is involved in ethanol-induced injury to the developing cerebellum (83). However, their results suggest Bax mediates the death of cerebellar Purkinje cells, but not CGC. Ethanol exposure during the peak period of cerebellar vulnerability resulted in a substantial loss of Purkinje cells in wild-type animals, but not in Bax knock-outs; CGC in Bax gene-deleted animals, however, are not similarly protected from ethanol effects. Overexpression of the anti-apoptotic Bcl-2 protein protects cerebellar Purkinje cells, but not CGC, against ethanol-induced death (84).

GSK3 β is also a critical mediator of intrinsic apoptotic signaling and is involved in neuroapoptosis (85). Recent evidence indicates that ethanol-induced activation of Bax and caspase-3 in the CNS of neonatal mice is GSK3 β -dependent (85). Lithium, an inhibitor of GSK3 β , protects ethanol-induced apoptosis of cultured CGC (86), suggesting that GSK3 β may be involved in ethanol-induced death of CGC.

Involvement of oxidative stress—The accumulation of intracellular reactive oxygen species (ROS) induces oxidative stress and causes mitochondrial membrane depolarization, which is followed by cytochrome c release, caspase activation, and apoptosis (87,88). The CNS is particularly susceptible to oxidative stress due to its high oxygen consumption rate, elevated levels of polyunsaturated fatty acids, and relatively low content of antioxidative enzymes. Oxidative stress has been proposed as a potential mechanism of ethanol-induced neurodegeneration in the developing, mature, and aging cerebellum (89).

Ethanol exposure at PD4 results in increased ROS (90) and decreased activity of antioxidative enzymes (81) in the cerebellum of rats. Interestingly, ethanol-induced ROS production in the neonatal cerebellum is observed in wild-type animals, but not in the Bax knock-outs (53). Chu et al. (2007) also demonstrate that prenatal exposure to ethanol induces mitochondrial damage, oxidative stress, and DNA damage as indicated by increased expression of 4-hydroxy-2,3-nonenal (HNE) and 8-OHdG in the cerebellum of neonatal rats (77). The *in vitro* results confirm that ethanol treatment impairs mitochondrial function and increases HNE and 8-OHdG immunoreactivity in cultured CGC. It appears that ethanol-induced oxidative stress and DNA damage is independent of its effects on insulin- or IGF-stimulated signaling (77). Smith et al. (2005) examine the effect of ethanol on concentrations of malondialdehyde (MDA) and reduced glutathione (GSH) which are indicative of oxidative stress (91). The rats received ethanol treatment from PD4 through PD9. Their study indicates that levels of MDA and reduced GSH in the cerebellum, but not in the hippocampus and cortex, are significantly elevated in animals receiving ethanol treatment.

The role of oxidative stress in ethanol-induced death of CGC is further explored by the treatment of antioxidants. Pycnogenol (PYC), a patented combination of bioflavonoids, is a potent antioxidant. PYC and the antioxidant vitamin E (VE) protect ethanol-induced apoptosis of cultured CGC in a dose-dependent manner (53). These results support the hypothesis that oxidative stress is involved in ethanol-induced death of CGC. Nevertheless, some evidence is not in favor of this hypothesis. Kane et al. (2008) evaluate the effect of *in vivo* ethanol exposure on CGC *in vitro*. In this model, ethanol was administered to rats *in vivo* via gavage on PD4 (vulnerable period) and PD14 (resistant period) (92). CGC were isolated 2-24 hours post-treatment, and ROS production and relative mitochondrial membrane potential (MMP) were immediately assessed in viable cells. The results show that ethanol exposure during either vulnerable or resistant periods does not induce CGC to produce ROS, it does not cause deterioration of neuronal MMP, and it does not cause neuron death. The mechanisms underlying this discrepancy are unclear. This may be due to the difference in sampling strategy or the process of tissue isolation.

Thiamine deficiency and protein translation inhibition—Thiamine, also known as vitamin B1, is an essential nutrient and plays an important role in metabolic and cellular function. Ethanol exposure is associated with thiamine deficiency (TD) (93,94). The cerebellum is particularly susceptible to TD (95), and it is thought to contribute to ethanol-associated cerebellar degeneration (89).

Mulholland et al. (2005) use organotypic cerebellar slice cultures to investigate the effect of ethanol and TD on cerebellar cytotoxicity (96). They show that neither 11-day ethanol treatment nor withdrawal from 10-day exposure significantly increased cerebellar cytotoxicity in cerebellar slices, as measured by propidium iodide fluorescence. TD significantly increases cerebellar cytotoxicity 21% above levels observed in control tissue. Cultures treated with both ethanol and TD display a marked increase in cytotoxicity approximately 60-90% above levels observed in control cultures. TD- and ethanol-induced

cytotoxicity is prevented in cultures co-exposed to thiamine. These findings suggest that the interaction between TD and ethanol causes much more damage to the cerebellum.

Similarly, in primary cultures of CGC, ethanol and TD act in synergy to induce much greater apoptosis than treated with either ethanol or TD alone (97). In parallel, ethanol synergizes TD-induced activation of double-stranded RNA (dsRNA)-activated protein kinase (PKR). PKR, a serine/threonine protein kinase, plays an important role in protein synthesis and cell survival. PKR has been well known for its anti-viral response. Upon activation by viral infection or dsRNA, PKR phosphorylates its substrate, the alpha-subunit of eukaryotic translation initiation factor-2 (eIF2 α) leading to inhibition of translation initiation. In the absence of a virus or dsRNA, PKR can be activated by direct interactions with its protein activators, PACT, or its mouse homologue, RAX. Both ethanol and TD activate PKR/eIF2 α in the cerebellum and cultured CGC (98,99). A selective inhibitor of PKR ameliorates TD- and ethanol-induced death of CGC in culture (97). High expression of RAX is observed in the developing cerebellum during the period of peak vulnerability to ethanol, and ethanol promotes PKR/RAX interaction (98). Ethanol-mediated apoptosis and the inhibition of protein synthesis positively correlate to the expression of RAX in cultured neuronal cells. Inhibition of RAX function by a dominant-negative protein abolishes ethanol-induced PKR activation, protein synthesis inhibition, and apoptosis (98). These results suggest that ethanol may interact with TD, resulting in PKR/RAX-dependent inhibition of protein synthesis and apoptosis in CGC.

Modulation of potassium channel currents—There is compelling evidence that K⁺ channels play an important role in the control of programmed cell death. Primary cultures of CGC have been shown to possess various voltage-gated outward K⁺ currents, including fast transit outward I_A and delayed rectifier K⁺ current (I_K) (100,101). Lefebvre et al. (2009) demonstrate that at low (10 mM) and high (400 mM) concentrations, ethanol evokes membrane depolarization and hyperpolarization in CGC, respectively (102). Ethanol (10 mM) depresses the I_A potassium current whereas ethanol (400 mM) provokes a marked potentiation of the specific I_K current. Ethanol (400 mM) induces pro-apoptotic responses whereas ethanol (10 mM) promotes cell survival. Previous studies have shown that a high KCl concentration (depolarizing condition) promotes CGC survival while a low KCl level induces CGC death (55). Neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) prevents CGC from apoptotic cell death induced by ethanol through at least in part, inhibition of I_K current (51,101). These results indicate that in CGC, ethanol mediates a dual effect on K⁺ currents partly involved in the control of CGC death.

3. Effect of ethanol on other developmental events

In addition to inducing neuroapoptosis, ethanol may deplete CGC by reducing the pool of CGC precursors (CGCP) in the EGL. CGCP can be isolated from PD2-3 rat cerebellum. These cells proliferate *in vitro* and offer an excellent system to study the effect of ethanol on cell cycle kinetics (103). Cyclin-dependent kinases (CDK) and their inhibitors (CKI) play an essential role in controlling cell cycle progression. We have demonstrated that ethanol alters the CDK system and disrupts cell cycle kinetics of CGC precursors in culture (103). The duration of the S-phase and total cell cycle is significantly prolonged by ethanol exposure by 220% and 135%, respectively, while the growth fraction is decreased from 44% in the control groups to 22% in the ethanol-exposed cultures (103). These findings are confirmed in an animal study showing that prenatal exposure to ethanol alters the CDK system in the developing cerebellum (104).

Ethanol may also inhibit the differentiation of CGC. Ethanol inhibits neurite outgrowth of CGC in culture (51,105). It appears whether ethanol inhibits differentiation or induces

apoptosis depends on the concentration of ethanol administered. For example, exposure of 7-day-old rat pups to ethanol for 3 hours moderately increases BAC (approximately 40 mM) and inhibits neurite formation and the activation of a Rho family of small GTPases (Rac1) in CGC. Longer exposure to ethanol for 5 hours results in higher BAC (approximately 80 mM), induces apoptosis, and inhibits Rac1 (44).

During the postnatal morphogenesis of the cerebellar cortex, CGC in the EGL migrate through an already developed ML and PCL to reach their final destination in the IGL where they differentiate into mature neurons (106). Ethanol exposure during this period inhibits the migration of CGC from the EGL to the IGL (107). Interestingly, prenatal ethanol exposure also delays the migration of CGC during the postnatal period, indicating that prenatal exposure to ethanol is sufficient to disrupt the neuronal migration program (108). Similarly, de la Monte et al. (2009) show CGC isolated from prenatal rats exposed to ethanol display impaired migration *in vitro* (109).

4. Neuroprotection against ethanol-induced death of CGC

In rodent cerebellum, the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is expressed by Purkinje cells and its receptors are present in CGC during both development and adulthood. PACAP regulates the development of CGC and most of the neurotrophic effects of PACAP are mediated through the cAMP/PKA and PI3K signaling pathways (110,111). PACAP prevents ethanol-induced death of CGC in culture and inhibits the expression of apoptotic markers (51). Neuroprotection by PACAP *in vitro* is confirmed by an animal study showing the ability of PACAP to counteract ethanol toxicity in 8-day-old rats (112). PACAP blocks ethanol-induced reduction of CGC in the IGL and ameliorates ethanol-induced expression of pro-apoptotic genes including c-jun or caspase-3. Behavioral studies further reveal that PACAP reduces the deleterious action of ethanol in the negative geotaxis test (112). However, other reports show ethanol exposure accelerates the anti-apoptotic effect of PACAP on cultured CGC (110). PACAP protects CGC cultured in the presence of 5 mM KCl against spontaneous apoptosis, and ethanol exposure accelerates the anti-apoptotic effect of PACAP by a mechanism that involves the PKA and PI3K pathways (110). The discrepancy of these studies may result from differences in ethanol concentrations and the age of CGC cultures applied.

Neurotrophins and growth factors are also potential neuroprotective agents. Luo et al. (1997) demonstrate that basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) significantly reduce the ethanol-induced loss of CGC in culture (41). Neuronal nitric oxide synthase (nNOS)-mediated signaling pathway involving nitric oxide (NO), the NO-cyclic GMP (cGMP), and cGMP-dependent protein kinase (PKG) plays a role in the acquisition of ethanol resistance by CGC (113). Bonthius et al. (2003) show that bFGF, NGF, and IGF-1, but not BDNF, utilize the NO-cGMP-PKG pathway to signal neurotrophic and neuroprotective effects against ethanol toxicity in CGC cultures (114).

L1 cell adhesion molecule (L1), a protein critical for appropriate development of the CNS, is a target for ethanol teratogenicity. Ethanol inhibits both L1 mediated cell adhesion as well as L1 mediated neurite outgrowth (115). A recent study shows that L1 may regulate neuronal survival, and CGC grown in L1 substratum are more resistant to ethanol-induced cell death (116). Other agents also exhibit a neuroprotective property and ameliorate ethanol-induced death of CGC. These include nicotine, gangliosides, and antioxidants such as vitamin E. They reduce ethanol-induced expression of apoptotic markers in cultured CGC in a concentration-dependent manner (50,53,54).

Conclusions

Ethanol exposure induces apoptotic death of CGC in the developing cerebellum. In general, *in vitro* studies using primary cultures of CGC confirm this finding. The mechanisms of ethanol-induced CGC death are complex and likely reflect the combined outcomes of promoting intrinsic apoptotic pathways and inhibiting anti-apoptotic signaling. Multiple mechanisms may interplay; these include inhibition of NMDA receptors, interference with signaling by neurotrophic factors, induction of oxidative stress, modulation of retinoid acid signaling, disturbance of potassium channel currents, thiamine deficiency, and disruption of translational regulation. Some important issues, however, remain controversial and require further investigation. For example, ethanol is known to produce ROS in the brain. Evidence, both pro and con, regarding the involvement of oxidative stress in ethanol-induced CGC death has been reported. Further investigation using a more sensitive oxidative stress detection system and consistent paradigms of ethanol exposure and sampling is necessary to resolve the controversy. In summary, the study of ethanol-induced damage to CGC provides important insight into the mechanisms of structural anomalies caused by fetal alcohol exposure. The information will be invaluable for the development of interventional and therapeutic strategies.

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Abbreviations

BAC	blood alcohol concentration
BDNF	brain-derived neurotrophic factor
CDK	cyclin-dependent kinase
CGC	cerebellar granule cells
CGCP	CGC precursor
CNS	central nervous system
EGL	external germinal layer
FASD	Fetal Alcohol Spectrum Disorders
IGF-I	insulin-like growth factor I
IGL	internal granule layer
ML	molecular layer
MMP	mitochondrial membrane potential
NGF	nerve growth factor
nNOS	neuronal nitric oxide synthase
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NT-3	neurotrophin-3
PACAP	pituitary adenylate cyclase-activating polypeptide
PCL	Purkinje cell layer

PD	postnatal days
PI3K	phosphatidylinositol 3-kinase
PKG	cyclic GMP-dependent protein kinase
PKR	double-stranded RNA-activated protein kinase
RA	retinoic acid
ROS	reactive oxygen species
TD	thiamine deficiency

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