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Alcohol, Astroglia, and Brain Development

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Abstract

Glial cells constitute one of the most common cell types in the brain. They play critical roles in central nervous system (CNS) development. Recent evidence demonstrates that glial cells are profoundly affected by prenatal alcohol exposure, suggesting that alterations in these cells may participate in CNS abnormalities associated with ethanol-induced teratogenesis. In vivo studies show that prenatal exposure to alcohol hampers myelinogenesis and is associated with neuroglial heterotopias and abnormal astrogliogenesis. Studies using primary cultures of rat cortical astrocytes show that ethanol affects DNA, RNA, and protein synthesis, decreases the number of mitotic cells, alters the content and distribution of several cytoskeletal proteins including the astroglial marker, glial fibrillary acidic protein (GFAP), and the levels of plasma-membrane glycoproteins, reduces the capacity of astrocytes to secrete growth factors, and induces oxidative stress. Furthermore, ethanol exposure during early embryogenesis alters the normal development of radial glia cells (the main astrocytic precursors), delays the onset of GFAP expression, and decreases mRNA GFAP levels in fetal and postnatal brains and in radial glia and astrocytes in primary culture. Recent evidence suggests that ethanol interferes with the transcription process of GFAP, thus leading to a reduction in GFAP-gene expression during astrogliogenesis. However, brief exposure of rats to high levels of ethanol during the neonatal period (the period of astrocyte differentiation) causes a transient gliosis, with an increase in GFAP and its mRNA levels. These findings indicate that astroglial cells are an important target of ethanol toxicity during central nervous system (CNS) development.

Index Entries: Ethanol; astrogliogenesis; radial glia; brain development; glial fibrillary acidic protein.

Introduction

One of the pioneers in demonstrating that the central nervous system (CNS) is made up not only of neurons but also of a large population of glial cells was Ramón y Cajal (Ramón y Cajal, 1911). Since that time it has become clear that glial cells are common cell types in the brain in many species, including humans (Reichenbach, 1989). Although during development the glia

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are the first cells to be formed, the vast majority do not arise until very late, after most of the neurons have developed, around the time of birth or in the early postnatal period. Indeed, one of the first direct evidence demonstrating that a specialized type of glial cell, the radial glia, coexists from the onset of neurogenesis was by the introduction of glial acidic protein (GFAP) as the glial-specific cell marker (Bignami and Dahl, 1974). Since then, numerous cell-specific molecular markers have confirmed that glial cells are present during the entire course of neural development (Cameron and Rakic, 1991) and it has gradually been established that glial cells play critical roles in the adult and developing central and peripheral nervous system. For example, it has been suggested that contact between neurons and glia in the embryo control the number of various kinds of cells, and determines their correct proportion. Such contacts are essential for neural migration and in the guidance of growth cones. Glial cells are also thought to be essential metabolic intermediaries between the endothelial cells of the capillaries and neurons alone or to participate in the blood–brain barrier (reviewed in Rakic, 1991). The processes of glial cells are also implicated as intermediary elements in the rearrangements and elimination of synaptic junctions and synaptic plasticity. Finally, glial membranes are recognized as being actively involved in the exchange of nutritients, neurotransmitters, second messengers, ions, and various growth factors (e.g., Müller et al., 1994; Hansson and Rönnbäck, 1995), all of which are critical processes of normal brain development. Indeed, several lines of evidence indicate that genetically or environmentally induced disturbances of glial cells or neuronal-glial interactions during critical periods lead to various molecular, structural, and functional abnormalities of the brain (Norenberg et al., 1988). Astroglial pathology appears to be a common response to ethanol toxicity in the adult central nervous system (Fein et al., 1994) and recent evidence demonstrates that ethanol exposure during brain development disrupts the proliferation and differentiation of astroglial cells (Miller, 1992; Guerri et al., 1993). Our main goal is to review recent cellular and molecular studies from different laboratories that support the hypothesis that astrocytes are among the main targets of ethanol toxicity during CNS development, and that alterations in these cells may underlie the CNS dysfunctions associated with ethanol-induced teratogenesis. Although in this article we focus exclusively on astrocytes, other glial cells are also affected by ethanol and these have been reviewed elsewhere (Phillips, 1992, 1994).

Ethanol and the Developing Brain: The Fetal Alcohol Syndrome

In the past 20 yr it has been clearly established that ethanol is an important teratogen and its consumption during pregnancy induces harmful effects on the developing fetus that lead to the fetal alcohol syndrome (FAS) (Clarren and Smith, 1978). However, CNS dysfunctions are the most distressing and permanent consequence of maternal consumption of alcohol. These dysfunctions can occur in absence of gross morphological defects associated with FAS (Streissguth et al., 1994). The mechanisms underlying the deleterious effects of alcohol on the developing nervous system are not completely understood. Clinical and experimental evidence has shown that ethanol exposure during brain development induces a variety of disruptions in normal neuronal development patterns, including depression of neurogenesis, delayed and aberrant neuronal migration (Miller, 1992), changes in the ontogeny of neurotransmitter synthesis (Druse, 1992), and neuronal depletion in a number of regions (Bonthius and West, 1991).

Abnormalities in glial development have been also suspected to contribute to the adverse effects of ethanol on the developing brain, since neuroglial heterotopias, agenesis of the corpus callosum and anterior commissure (areas originally formed by neuroglial cells), ectopic clusters of neurons and glial cells

near the pial surface have been observed in postmortem studies of FAS children (Claren et al., 1978; Peiffer, 1979). In addition, smaller white-matter areas and abnormalities of the corpus callosum have been noted in brains of FAS patients examined with magnetic resonance imaging (Riley et al., 1995; Swayze et al., 1997), which indicates that ethanol affects glia and myelin development, as well as axon development.

Studies on experimental animals exposed to alcohol during development have also provided evidence of glial involvement in alcohol-induced CNS alterations. For example, one of the developmental stages most vulnerable to ethanol-induced reductions in brain size in experimental animals is the brain-growth spurt (Bonthius and West, 1991), a period of major development of glial and myelin structures, and this suggests a potential effect of ethanol on glial cells.

Studying the mechanisms by which alcohol alters the development of the CNS and the cellular and molecular basis of those alterations is important to better understand the specific roles of each cell type in the response of the developing CNS to the toxic effect of ethanol.

Alcohol and Astroglial Cells in Culture

Because of the difficulties involved in analyzing the direct toxic effect of ethanol on astrogliogenesis, astroglial cells* in primary culture have been widely used. From these studies it was possible to demonstrate that astroglial cells in culture have a greater sensitivity to alcohol than neurons (Lokhorst and Druse, 1993; Ledig and Tholey, 1994), and that even moderate levels of alcohol delayed growth and maturation of these cells (Renau-Piqueras, 1989; Davies and Cox, 1991; Ledig and Tholey, 1994). Two main types of approaches have been used to study the effects of alcohol on astroglial cells: first, the addition of different concentrations of ethanol to the cell culture medium; and second, the culture of cells from brains of newborn animals exposed to alcohol during gestation (via maternal exposure). These cultures were carried out in the absence of alcohol in the medium. In some of our studies the latter approach has been used, but with the variant that astroglial cells have been isolated from fetuses of chronic alcohol-fed rats in which the alcohol was administered with a liquid diet before and during gestation (prenatal alcohol exposed, or PEA, cells [Renau-Piqueras et al., 1989]). During this alcohol treatment, the blood-alcohol levels reached by the alcohol-fed pregnant rats and their fetuses were 105 ± 40 and 115 ± 20 mg/dL (Sanchis et al., 1986). Using the offspring of chronic alcohol-fed animals we have reproduced in the rat (Guerri et al., 1984; Esquifino et al., 1986; Portolés et al., 1988; Renau-Piqueras, 1991) many of the alterations observed in children with FAS (Clarren and Smith, 1978). The advantage of the PEA astroglial cells is that they permit assesment of the effect of ethanol on astrocyte-precursor cells, which appear during the embryonic stage of brain development (Cameron and Rakic, 1991). Regarding the possible drawbacks associated with this model, we have only observed some alterations in the ability of PEA cells to adhere to the culture substrate, which may result in a decreased number of cells.

Effect of Alcohol on Cell Growth

Ethanol at physiologically relevant and at high concentrations has been shown to produce various effects on the synthesis of DNA, RNA, and protein in primary culture of astroglial cells. Low in vitro ethanol concentrations (11 m*M*) increase DNA, RNA, and protein synthesis (Kennedy and Mukerji, 1986), whereas higher concentrations (25–200 m*M*) or *in utero* exposure to alcohol inhibit these parameters that affect both cell proliferation and differentiation (Kennedy and Mukerji, 1986; Guerri et al.,

* In general, we will use the term astroglia for cells in culture and the term astrocyte for cells *in situ* within the CNS.

1990). The inhibition of DNA synthesis induced by high ethanol levels could be the result of an accumulation of cells in the G_0/G_1 phase of the cell cycle, which would avert the normal evolution of cells toward the S phase and decrease the number of mitotic cells (Guerri et al., 1990). Ethanol-induced alterations in cell division machinery resulting from the inhibition of cytoskeleton assembling (Sáez et al., 1991) or an effect of ethanol on some of the cyclins (e.g., D1 or D2) (Mikami et al., 1997) may be involved in these effects.

Ethanol at high concentrations (> 50 m*M*) or prenatal alcohol exposure also impairs astroglia proliferation (Davies and Vernadakis, 1984; Guerri et al., 1990; Davies and Cox, 1991), inhibiting RNA and protein synthesis (Guerri et al., 1990) and delaying the morphological maturation and the levels of astrocyte-specific markers such as glutamine synthetase (GS) (Davies and Vernadakis, 1984) and GFAP (Renau-Piqueras, 1989; Saez et al., 1991). However, in some studies, no changes in the levels of GS or GFAP were found in astroglial cells exposed in vitro to alcohol (Chiappelli et al., 1991). Although the mechanism of the ethanol-induced inhibition of protein synthesis is unknown, it has been suggested that ethanol inhibits the amino acids transporters (Snyder et al., 1994). In addition, in PEA astroglial cells the decrease in ^{3}H-leucine was accompanied by a diminution in the total RNA and mRNA content in these cells, suggesting that ethanol interferes with protein synthesis at the transcription levels (Guerri et al., 1993). Indeed, in vitro protein synthesis experiments demonstrated no differences in the amount and pattern of proteins synthesized by control and PEA cells, when the same amount of RNA from control or PEA cells was used (Guerri et al., 1993). Recent evidence also indicates that ethanol depresses the proliferative activity of glial cells by interfering with the stimulatory effect of trophic factors (e.g., basic fibroblast growth factor, insulin-like growth factor or IGF I) (Snyder et al., 1994; Luo and Miller, 1996). In summary, results from several studies indicate that in vitro exposure to high concentrations of ethanol or prenatal alcohol exposure impairs growth and differentiation of astroglial cells, although further studies are needed to clarify the molecular mechanisms involved in these effects.

Morphological Changes Induced by Ethanol in Atroglial Cells

Ethanol exposure also induces important morphological modifications in astroglial cells that in vivo and in primary culture can be related to biochemical, cytochemical, immunocytochemical, and functional alterations. Studies *in situ* show that in cerebellum, one of the most striking aspects of the neurophil adjacent to the larger dendritic processes was the distended, watery appearance of the glial wrappings (Tavares and Paula-Brabosa, 1984; Popova and Shchekalina, 1980; Smith and Davies, 1990). Alcohol-induced swelling of astroglia cells have also been demonstrated using primary cultures (Kimelberg and Aschner, 1994). Moreover, in optic nerve sections from rats prenatally exposed to alcohol, astrocyte displayed ultrastructural alterations including altered mitochondria and dilated endoplasmic reticulum (Pinazo-Duran et al., 1993).

A direct effect of alcohol on the morphology of astroglial cells in culture has also been observed. Thus, astroglial cultures showed an alcohol dose-dependent attenuation in both the depth and complexity of the cell layer that indicated that growth kinetics and morphological development of astrocyte cultures are vulnerable to alcohol exposure (Davies and Cox, 1991). In addition, PEA astroglial cells show important qualitative and quantitative ultrastructural changes, including alterations in several cell components such as lysosomes, mitochondria, rough endoplasmic reticulum, and Golgi apparatus (Mayordomo et al., 1992). The morphological alterations in the last of these cell components were accompanied by variations in the cytochemical activity of enzymes located in these organelles (e.g., acid phosphatase, thiamine pyrophosphatase, uridine diphosphatase, and so on), suggesting an alteration

of the functional state of the Golgi apparatus (Mayordomo et al., 1992). Stereological analysis of the cell-surface components (microvilli, coated pits, uncoated pits) indicated that prenatal exposure to ethanol also alters the surface topography of proliferating and differentiating astroglial cells (Renau-Piqueras et al., 1992). These alcohol-induced alterations in the plasma membrane occurred together with a decrease in the ability of these cells to bind concanavalin A, and a decreased activity of several membrane-bound enzymes, including 5'-nucleotidase and (Na-K)ATPase (Renau-Piqueras et al., 1992), suggesting that alcohol could alter not only the plasma membrane functions, but also the glycosylation and transport of proteins, as occurs in adult and fetal hepatocytes (Renau-Piqueras et al., 1997). In fact, it has been demonstrated that prenatal exposure to alcohol increases the intracellular pool of both nerve growth factor (NGF) and its receptor (NGFr) in the cytoplasm of astroglial cells, and that these changes were accompanied by an important reduction in the secretion of this important growth factor (Vallés et al., 1994). These results suggest that alcohol affects the secretory process, perhaps as a result of an alcohol-induced alteration of the cytoskeletal elements (*see* Astroglial Cytoskeleton) involved in intracellular protein trafficking. Ethanol-induced alterations in the Golgi apparatus (Mayordomo et al., 1992), glycosylation, and recycling (Renau-Piqueras et al., 1997) processes might also participate in the above effects. In fact, work in progress in our laboratory indicates that alcohol also alters endocytosis and translocation of macromolecules in astroglial cells.

Astroglia are known not only to release NGF (Furukawa et al., 1986) but also to synthesize and secrete a number of growth factors (Müller et al., 1994) that are essential for normal neural development. Recent evidence demonstrates that ethanol inhibits the production of essential neurotrophic factors (Heaton et al., 1992). Altered production of these factors is particularly important because of the evidence that astroglial factors promote the development of the differentiated state of catecholaminergic (Lieth et al., 1989) and serotonergic neurons (Whittaker-Azmitia and Azmitia, 1989), both of which are adversely affected by *in utero* ethanol exposure (Druse, 1992). In fact, it has been shown that ethanol-exposed astrocytes produce and/or secrete less of the trophic factors that are essential for normal development of serotonergic neurons (Whittaker-Azmitia et al., 1996). Alteration in astroglial growth factors may influence neurogenesis, migration, and axonal guidance, and therefore brain development.

Astroglial Cytoskeleton

One of the most characteristic features of astrocytes is their cytoskeleton (CSK), which appears not only to perform important structural functions, including the formation of a framework for migration of neurons during development, but also to be involved in cell motility, mitosis, trans- and intracellular transport, adhesion, modulation of membrane activity, and cellular morphogenesis (Fuchs and Weber, 1994). Ethanol exposure significantly alters the CSK components of the astroglial cells. Thus, both in vivo prenatal alcohol exposure (Sáez et al., 1991; Gressens et al., 1992) and in vitro alcohol treatments reduce the content and distribution of GFAP, the main intermediate filament protein of astrocytes and a marker for these cells (Renau-Piqueras, 1989; Davies and Cox, 1991). Furthermore, a decrease in the content of other cytoskeletal proteins such as vimentin and tubulin has also been observed (Sáez et al., 1991). Immunofluorescence and immunogold electron-microscopy studies of GFAP also indicate that ethanol-exposed astrocytes failed to develop processes or acquire a filamentous intermediate filament (IF) distribution pattern. In addition, PEA cells showed some cytoplasmic areas lacking CSK elements and others with IF displaying an abnormal distribution in bundles or in balls (Fig. 1) (Sáez et al., 1991). These results indicate that alcohol exposure, either *in utero* or in vitro, induces alterations in astrocyte development. These findings raise the question as to whether the

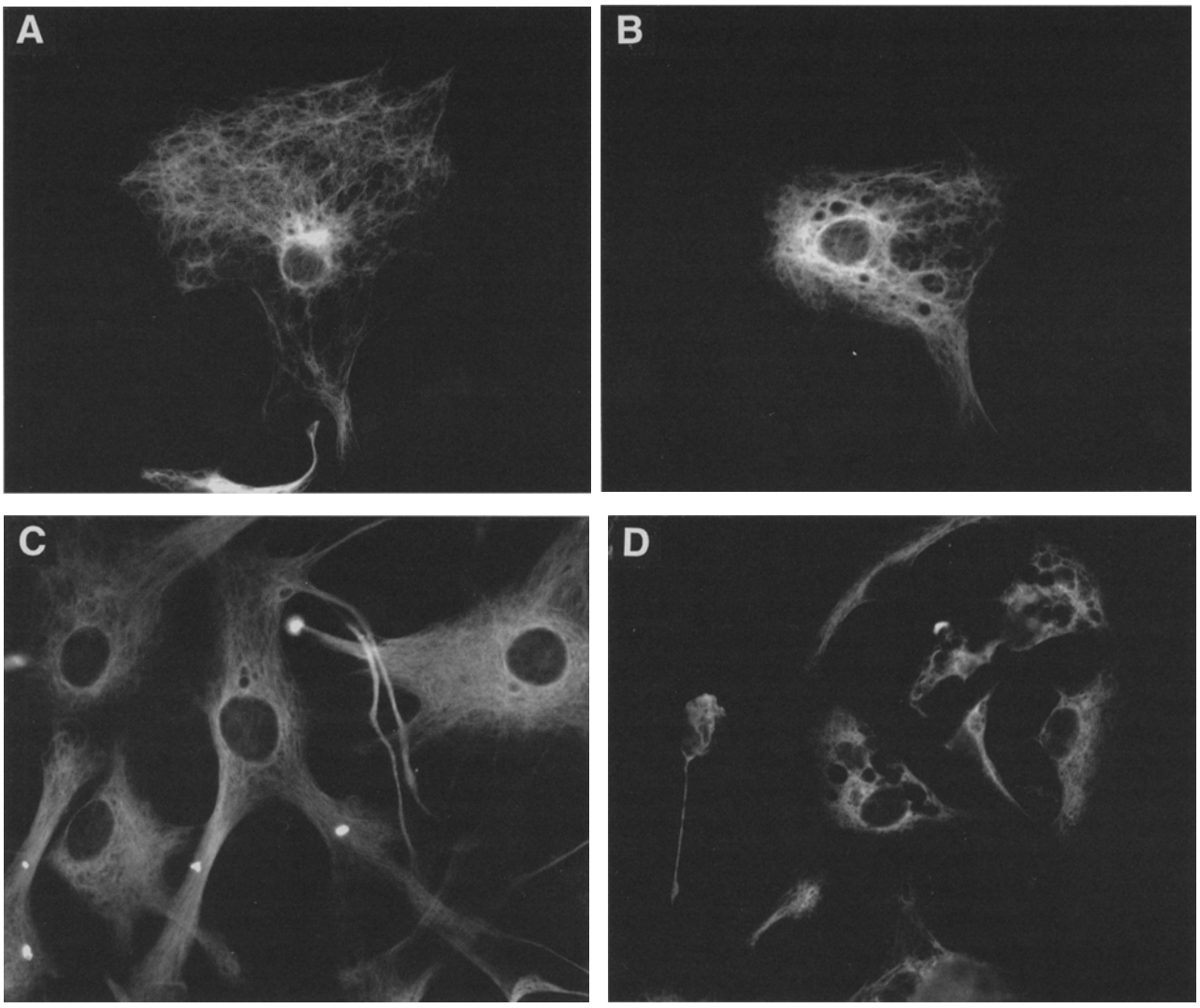

Fig. 1. Immunofluorescence of GFAP of cortical astrocytes at 4 **(A,B)** and 10d **(C,D)** in primary culture. Control astrocytes (A,C) and cells from animals exposed to alcohol during gestation (via maternal exposure) and cultured in absence of alcohol (B,D). As illustrated, alcohol alters both the cell morphology and the GFAP distribution pattern that is manifested by cytoplasmic areas lacking cytoskeletal elements and others with an abnormal distribution of filaments (B,D). Original magnification. A,B and D, ×650; C, ×700.

effects observed in astroglial cells in primary culture are also reflected in vivo. To address this question, we determined the levels of the astrocyte marker, GFAP (Eng et al., 1971), in the brains of pups from alcohol-fed mothers. In the brains of control animals, the levels of this protein increase during the postnatal period, which correlates with astroglial development. However, in brains of pups from alcohol-fed mothers, a significant lower increase in the levels of GFAP and its mRNA (Vallés et al., 1997) was observed (Fig. 2) (Sáez et al., 1991). These results support the idea that alcohol exposure during brain development alters the expression of astroglial markers, which suggests an effect of alcohol on astroglial development. Consistent with these findings, a reduction (approx 30%) in the number of astrocytic cortical population

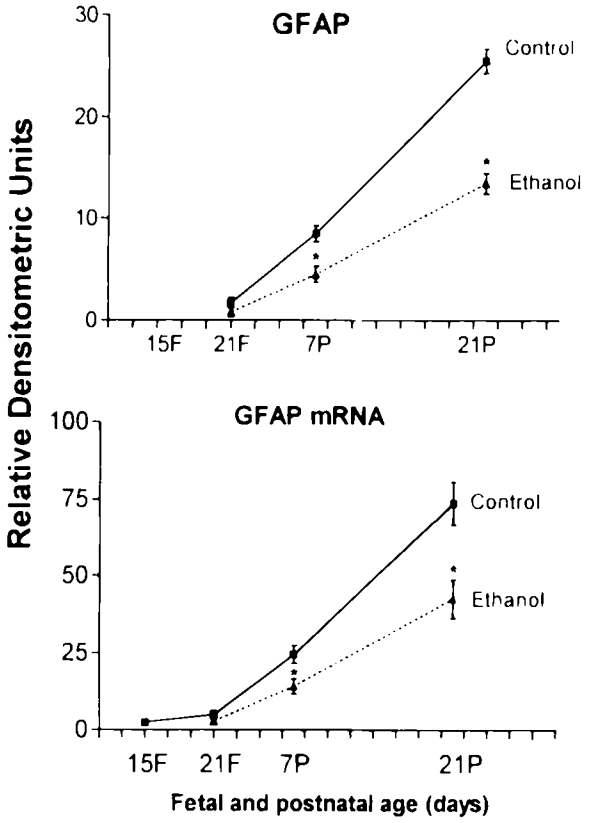

Fig. 2. Effect of prenatal exposure to alcohol on the developmental pattern of GFAP expression (protein and mRNA) during fetal and postnatal brain development. The GFAP protein and mRNA levels were determined in rat offspring brains of dams fed during gestation and lactation with control or alcohol-containing diet (5%, w/v). Blood alcohol levels reached by the alcohol-fed pregnant rats were 105 ± 40 mg/dL. Data represent the densitometric analysis obtained from the Western and Northern blotting of brains at different fetal (F) or postnatal (P) days. Constant protein and mRNA were used. Each value represents the average ± SD of five brains from different litters. *Significant different ($p < 0.001$) from control values.

in alcohol exposed pups was found in *in situ* studies (Gressens et al., 1992; Miller, 1992).

In contrast with the findings after prenatal alcohol exposure, chronic ethanol intake induces an increase in GFAP immunoreactivity (Franke, 1995), which is a hallmark of astrogliosis, a common reaction of astrocytes to injury in the mature nervous system (Hatten et al., 1991). Interestingly, brief postnatal (d 4–9) exposure to high levels of alcohol (175–300 mg/dL) can also cause transient astrogliosis manifested by an increase in immunoreactive GFAP, primarily in layer V of the cerebral cortex (Goodlett et al., 1993) that may result from gliosis around blood capillaries damaged by the high ethanol doses. However, an increase in GFAP mRNA levels has also been observed in cerebral cortex, but not in the hippocampus, cerebellum, or brainstem of neonatal rats and in confluent astroglia cells in culture (3 wk after plating) when exposed to high levels of alcohol (175–200 mg/dL) for a short period of time (Fletcher and Shain, 1993; Goodlett et al., 1993). These results suggest that the effect of ethanol on astrocytes, and specifically on GFAP, depends on the levels of alcohol, duration, and timing of exposure relative to the stage of glial maturation (glial progenitor cells, proliferation, or differentiation of astrocytes) (Fig. 3). In addition, the brain-regional differences in glial response to ethanol concerning the expression of GFAP may either reflect, as suggested by Fletcher et al. (1994) stage-dependent vulnerability of astrocytes (different brain regions perform specific developmental functions asynchronously) or heterogeneity of astrocytes within the different brain regions.

Changes in GFAP expression can occur at both transcriptional (Brenner, 1994) and translational levels (Inagaki et al., 1994). However, the molecular mechanisms involved in these changes during brain development, aging, or brain injury are largely unknown (Hatten et al., 1991; Brenner, 1994; Inagaki et al., 1994; Eng and Ghirnikar, 1994).

Results from our laboratory suggest that ethanol interferes with transcription, since the decrease in GFAP immunoreactivity is accompanied by a reduction in its mRNA level in both PEA astrocytes and brain of pups from alcohol-fed mothers. In fact, using run-off experiments from nuclei of control and PEA astrocytes, we could demonstrate that ethanol exposure significantly decreases the GFAP transcription rate and slightly reduces GFAP mRNA stability (Vallés et al., 1997). We further demonstrate that

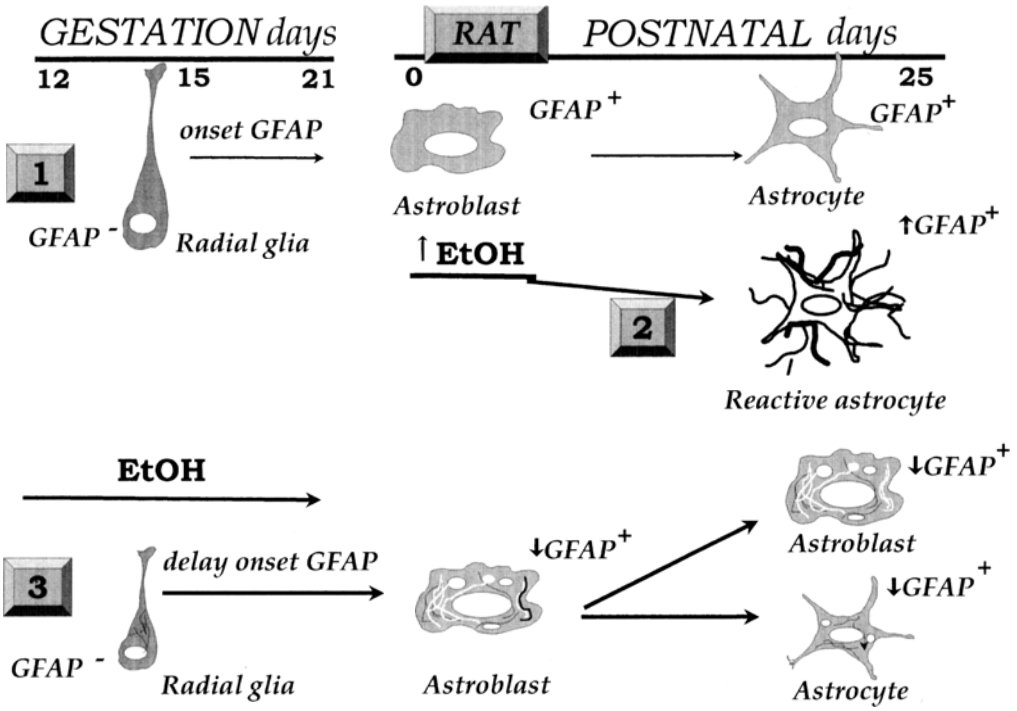

Fig. 3. Schematic representation illustrating that the effect of ethanol on GFAP depends on the levels of alcohol, duration, and timing of exposure relative to the stage of astroglial maturation in rat cerebral cortex. 1 Under normal conditions, radial glia, which appears at early neuroembryogenesis, is transformed into GFAP+ astroblasts and then into differentiated astrocytes. 2) Brief postnatal exposure to high levels of alcohol during astrocyte differentiation results in a transient astrogliosis. 3) Exposure to ethanol during gestation that includes early embryogenesis, alters the morphology of radial glia, delays the onset of GFAP and leads to damaged astrocytes.

the effect of alcohol on GFAP expression was specific for this gene, since the gene for vimentin, another astrocyte intermediate filament, was not altered by ethanol (Vallés et al., 1997).

Regulation of the GFAP gene seems to be quite complex, with multiple interacting DNA elements (Brenner, 1994). The transcription factor AP-1 and factors that mediate responses to cAMP and protein kinase C have been suggested to be involved in the regulatory events of GFAP expression during development (Riol, et al., 1992). In addition, GFAP promotor contains clusters of putative response elements for diverse hormones (e.g., glucocorticoids, sex hormones), cytokines, and growth factors that modulate GFAP transcription and expression (Laping et al., 1994). Ethanol has been shown to alter cyclic AMP (Pennington, 1988) and protein kinase C (Slater et al., 1993), and both kinases are involved in the transcription control of GFAP (Shafit-Zagardo, 1988; Kaneko et al., 1994). Ethanol exposure during fetal development also induces marked alterations in growth factors and hormones, including glucortocoids and sex steroids (Weinberg, 1994) that might also affect the expression and transcription of GFAP (Laping et al., 1994). In addition, ethanol-induced changes in fetal GFAP gene methylation could also be involved in the regulation of transcription and gene expression of GFAP (*see* Ethanol Induces Oxidative Stress in Astroglial Cells). Therefore, ethanol-induced alterations in some of the GFAP transcription modulatory elements or in the methylation process during critical periods of brain development (e.g., the onset of GFAP expression) may influence the transcription process leading to a reduction in the expression of GFAP.

Effect of Ethanol on Radial Glia

As described above, astroglial cells in primary culture derived from rats prenatally exposed to alcohol and cultured in the absence of this toxin show important morphological, biochemical, and functional alterations. Because the development of astrocytes in the rat mainly occurs during postnatal life, these results suggest that ethanol affects the precursor of astrocytes, the radial glial cells that appear during neuroembryogenesis. In fact, the presence of radial glia during early stages of the embryogenesis and their important role in neuronal migration are well documented (Cameron and Rakic, 1991; Hatten, 1990). In mammals, radial glia have a transient existence, because shortly after migration is completed, they are transformed into astrocytes (Cameron and Rakic, 1991). Radial glia cells express both vimentin and nestin during early stages of CNS development, and later they lose vimentin reactivity when they are transformed into GFAP-positive astrocytes (Cameron and Rakic, 1991; Sancho-Tello et al., 1995).

Since studies of the effect of alcohol on radial glia are scarce (e.g., Miller, 1992; Phillips, 1994), we have investigated whether ethanol affects these cells in cerebral cortex using two approaches. First, we analyzed the developmental pattern of vimentin and GFAP immunoreactivity and gene expression during fetal embryofetal brain development, and second, we used primary cultures of radial glia, which is a useful tool for analyzing the glial-cell differentiation and transformation into astrocytes (Culican et al., 1990; Sancho-Tello et al., 1995). Primary cultures of radial glia were prepared from 13-d control or alcohol-exposed rat fetuses.

Using these approaches, we demonstrated that, whereas GFAP immunoreactivity appeared late in gestation (fetal d 21) and on d 5 of culture in radial glia, its encoding mRNA was first detected on fetal d 15 and increased in content on fetal day 21 (Vallés et al., 1996) (Fig. 2). In contrast, the levels of vimentin and its mRNA were high at fetal d 15, but decreased on fetal d 21. Chronic *in utero* alcohol exposure delays the onset of GFAP expression, and significantly decreases the GFAP (protein and mRNA) levels in both fetal brain and in primary culture of radial glia (Vallés et al., 1996). Interestingly, vimentin, the other major intermediate filament protein in these cells, was not significantly affected by ethanol. Another important finding is that glial cells obtained from ethanol-exposed fetuses showed some morphological alterations throughout the culture interval, including shorter glial processes, delay in their transformation into astrocytes, and changes in the organization of GFAP-distribution pattern (Vallés et al., 1996). These results strongly suggest that alcohol exposure during early embryogenesis alters radial glia differentiation and its transformation into astrocytes, which may explain the alterations observed in prenatal alcohol exposure astrocytes.

Changes in DNA methylation have been proposed as a possible mechanism involved in the teratogenic effects of ethanol (Garro et al., 1991). In fact, several lines of evidence indicate that methylation of cytosine residues plays an important role in the regulation of mammalian gene expression, especially during embryogenesis and differentiation. During these stages most of the tissue-specific genes are almost fully methylated and undergo programmed active demethylation at the moment of activation and transcription (Eden and Cedar, 1994; Razin and Shemer, 1995). Thus, methylation changes in tissue-specific genes during embryogenesis is likely to lead to a derrangement of normal fetal gene expression and fetal development.

In an attempt to further explore the mechanism(s) involved in the ethanol-induced delay in GFAP expression, the influence of ethanol on GFAP gene methylation has been analyzed. The results demonstrate that, whereas in control brains, between fetal d 15 and fetal d 21, the GFAP DNA goes from a highly methylated state to a partially methylated condition and that these changes occur concomitantly with the onset of GFAP expression, in alcohol-exposed brains the GFAP DNA is highly methylated at both fetal days analyzed. This suggests a

reduction in the demethylation process manifested by the hypermethylated state of the GFAP DNA at fetal d 21 (Vallés et al., 1997). These findings are consistent with the delay in the onset of GFAP expression observed in alcohol-exposed fetuses and in culture of radial glia. In addition, changes in GFAP DNA methylation induced by ethanol also agree with the decreased GFAP transcription rate observed in PEA astroglial cells.

These results suggest that neural progenitor cells such as radial glia are the main target of ethanol toxicity. In fact, a delay in the postnatal maturation of Bergmann glia (radial glia in the cerebellum) (Shetty and Phillips, 1992) and a reduction in soma size and fiber number (Pérez-Torrero et al., 1997) of these cells have been observed in brain of rats exposed to alcohol during gestation. This treatment also induces alterations in cortical radial glia including decreased number of cells (Gressens et al., 1992) and alterations in its morphology and in the timing of the phenotypic transition of these cells into more typical GFAP-positive astrocytes (Miller and Robertson, 1993). The main consequences of these effects are a premature degradation of radial glia networks and a faulty migration of late-generated cortical neurons and granule cells, resulting in neuronal ectopias (Gressens et al., 1992; Miller, 1992; Miller and Robertson, 1993).

At present, our understanding of the sequence events and factors that regulate the transformation of radial glia into astrocytes and the modulation of GFAP expression during development is limited (Cameron and Rakic, 1991). However, the presence of certain growth factors (Kentroti and Vernadakis, 1997) and/or hormones may be involved in this transformation and also can modulate GFAP expression (Laping, et al., 1994). Therefore changes in these modulatory factors during critical periods of brain development (e.g., the onset of GFAP expression) might explain the different effects of alcohol. In our studies we have used brains (or astroglial cells) from offprings of chronic ethanol-fed mothers that showed several hormonal alterations during development (Esquifino, et al., 1986; Portolés et al., 1988). In conclusion, because during early embryonic development, radial cells seem to play an integral role in the migration and in the guidance of axons and growth cones during the formation of neural circuits (Hatten, 1990; Rakic, 1991), ethanol-induced changes in radial glial development may be involved in heterotopias and altered neurogenesis observed after prenatal ethanol exposure (Miller, 1992).

One observation that encourages the hypothesis that the alcohol-induced damage to radial glia is an important mechanism involved in the teratogenic effect of alcohol on brain is that the corpus callosum is defective or absent in children with fetal alcohol syndrome (Clarren et al., 1978; Peiffer et al., 1979; Riley et al., 1995; Swayze et al., 1997). During embryonic development, the corpus callosum is initially formed by fascicles of radial glia that support the growth axons from one side of the brain to the other (Norris and Kalil, 1991). However, corpus callosum does not form in congenital mouse mutants lacking this structure or when the radial glia "sling" at the cerebral midbrain is disrupted (Silver and Ogawa, 1983). Therefore, an alteration in radial glia could be involved in the agenesis and abnormaties of the corpus callosum noted in children of alcoholic mothers (Riley et al., 1995; Swayze et al., 1997).

Ethanol Induces Oxidative Stress in Astroglial Cells

Recent evidence has also shown that ethanol-induced oxidative stress in astrocytes may participate in the cytoskeletal and membrane alterations observed in cells exposed in vitro to alcohol (Montoliu et al., 1995). Indeed, during the last few years evidence has accumulated on the role of free radical formation and oxidative stress in the pathogenesis of alcohol-induced cell injury (Guerri et al., 1994; Nordman et al., 1992) as well as in the teratogenesis produced by ethanol (Guerri et al., 1994; Kotch et al., 1995). One of the mechanisms that has been suggested

to be involved in the alcohol-induced free radical formation is the participation of the metabolism of ethanol to acetaldehyde, mainly through the intervention of the ethanol-inducible form of cytocrome P-450, termed CYP2E1 (Albano et al., 1991). This isoenzymatic form of the cytocrome P-450 has an apparently high rate of oxidase activity that causes the formation of reactive oxygen species (ROS) during its catalytic cycle, and these are able to initiate lipid peroxidation (Ingelman-Sundberg, 1993). Although this isoenzymatic form of P-450 is located predominantly in the liver, recent reports demonstrate that CYP2E1 is also expressed in brain (Montoliu et al., 1994; Tindberg and Ingelman-Sundberg, 1996). Interestingly, chronic ethanol consumption increases both the content of CYP2E1 (Montoliu et al., 1994; Tindberg and Ingelman-Sundberg, 1996) and the formation of oxygen radical species (Montoliu et al., 1994) in the brain, leading to a depletion of some endogenous antioxidant compounds, such as glutathione (GSH). A decrease in the levels or activity of antioxidant molecules may increased the levels of ROS, thus resulting in oxidative damage in the brain (Montoliu et al., 1994). These findings raise the questions whether there is a selective expression of CYP2E1 in the different nervous cell types, and whether ethanol-induced ROS is related to the induction of this isoform and/or to the damage in the nervous cells. Using biochemical and immunocytochemical assays, we demonstrate that CYP2E1 is expressed in astroglial cells in culture (Montoliu et al., 1995) (Fig. 4), which suggests the participation of CYP2E1 in the ethanol-induced free-radical generation in astrocytes.

The expression of CYP2E1 and its induction by ethanol in astrocytes are of toxicological interest, not only because of the capacity of this isoenzyme to metabolize ethanol to acetaldehyde, which is a very toxic compound (Klassen et al., 1995), but also because of its role in the metabolic activation of a large number of toxicological compounds including acetominophen, solvents, nitrosamines, and so on (Ingelman-Sundberg, 1993). Another toxicological consequence of the induction of the CYP2E1 in astrocytes is that

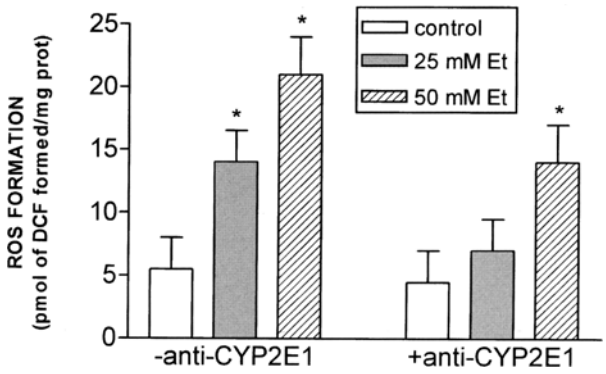

Fig. 4. Protective effect of CYP2E1 in the ethanol-induced formation of oxygen radical species (ROS) measured as the formation of the activated form of dichlorofluorescein diacetate. As shown, the incubation of cells with an anti-CYP2E1 antibody partially prevents the ROS formation induced by ethanol. Data are means ± SD (bars) values from five different determinations. *Significantly different ($p \leq 0.001$) from control values.

ethanol induces a dose-dependent increase in the formation of reactive oxygen species that leads to a GSH depletion and to oxidative stress in the cell (Montoliu et al., 1995). GSH is known to play a critical role in the cell detoxication process, including hydroperoxide catabolism, conjugation with electrophiles, and direct interaction of free radicals (Reed, 1990). Interestingly, it has been shown that astrocytes have much higher GSH levels than neurons (Makar et al., 1994; Raps et al., 1989). Neuronal GSH concentations have been reported to be dependent on the presence of glial cells providing neurons with the GSH precursor cysteine (Sagara et al., 1993). In addition, glutathione-*S*-transferase is located exclusively in glial cells, constituting a first line of defense against toxic sustances (Cammer et al., 1989). Therefore, since astroglial cells play an important role in the defense of the brain against reactive oxygen species (Makar et al., 1994), a depletion of GSH or other antioxidant systems induced by alcohol could influence the GSH availability to neurons, thus making these cells more susceptible to the toxic effect of ethanol. Finally, ethanol also decreases the content of other antioxidant systems

in astrocytes, such as formaldehyde dehydrogenase, which metabolizes formaldehyde, an important genotoxic compound generated during lipid peroxidation (Iborra et al., 1992).

The final question to be answered is whether or not the oxidative stress induced by ethanol in astrocytes is related to cell damage. Oxidative stress is known to be associated with damage to several cell components, including proteins and nucleic acids, and with structural damage in cytoskeleton and cell membranes (Orrenius, 1993) and these cell components are altered by ethanol. To explore this issue, cells were incubated with ethanol and cysteine or cystine, which increased the cellular synthesis of GSH (Sagara et al., 1993). By restoring the GSH levels in cells treated with ethanol, ethanol-induced decrease in GFAP was prevented (Fig. 5) (Montoliu et al., 1995). These results suggest that ethanol-induced oxidative damage in astrocytes may be involved in some alterations, such as cytoskeleton damage, observed in astrocytes exposed in vitro to alcohol. However, it remains to be demonstrated whether this mechanism also participates in alcohol-induced damage to radial glia and, in fact, an increase in oxygen radical species have been observed in the brain of fetuses from alcohol-fed mothers (Guerri et al., 1994).

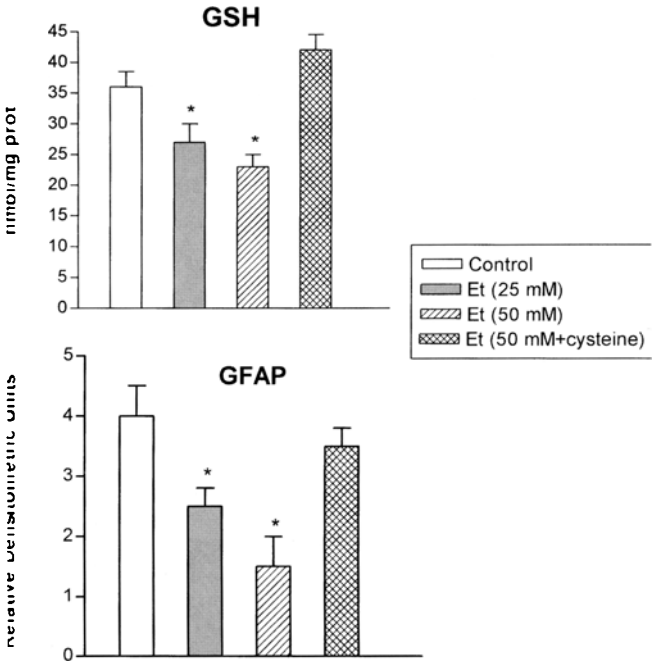

Fig. 5. Graphs illustrating the effect of alcohol on GSH and GFAP levels and the protective effect of the GSH on the alcohol-induced decrease in GFAP content in astrocytes. Control cells were incubated for 14 d with several concentrations of alcohol. In some experiments, cells were grown in a medium containing ethanol (50 mM) and cysteine (0.02 mM). Data are mean ± SD (bars) values from six different experiments. *Significantly different ($p \leq 0.002$) from control values.

Conclusions

Although glial damage is a dominant feature of the alcohol-induced brain injury observed in both chronic alcoholics and in children exposed to alcohol *in utero*, only recently have glial cells received attention in the study of brain response to alcohol (Lancaster, 1994). Evidence from in vivo and in vitro studies clearly supports the conclusion that ethanol alters astrogliogenesis in humans and experimental animals, by affecting key astroglial functions. Astroglial damage would be likely to have profound effects on many developmental processes in the CNS including, boundary formation during neural morphogenesis, cerebral compartmentalization, neuronal proliferation and migration, axon outgrowth, and guidance and availability of trophic support molecules. Ethanol can also disturb those astroglial functions involved in neuronal physiology and survival such as control of extracellular ion concentration, uptake, and inactivation of excitatory amino acids and other neunotransmitters. Therefore, alcohol-induced astrocyte damage could be a potentially important mechanism involved in the CNS dysfunctions observed after *in utero* alcohol exposure. However, it remains to be determined if the toxic effects of ethanol reviewed here are the result of several independent mechanisms or, if there is a single primary target (e.g., alterations of some specific genes) followed by a cascade of secondary effects. Recent findings indicate that

early exposure to alcohol profoundly affects radial glia, the precursor cell of astrocytes, altering the timing and extent of expression of the gene for GFAP and the content and organization of this protein that is the main cytoskeletal protein of astrocytes and a marker for these cells. Changes in the cytoskeletal proteins may impair cell proliferation, protein transport, and the secretion by astrocytes of factors involved in neuronal migration (e.g., cell adhesion molecules) and survival (e.g., NGF and other neurotrophins). GFAP is required for the formation of stable astrocytic processes in response to neurons (Weinstein et al., 1991), and GFAP gene expression was shown in mice carrying a null mutation to be essential for normal white-matter architecture and blood–brain barrier integrity (Liedtke et al., 1996). Thus, the structural support of astrocyte processes by GFAP can be expected to be critical for the morphogenesis of the CNS. Clearly, further research will be required to determine the relationship between ethanol, alteration in gene expression, and astroglial damage and to define how injury to these cells contributes to the toxic effects of ethanol on the developing nervous system.

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References

Albano, A., Tomasi A., Persson J-O., Terelius Y., Goria-Gatti L., Ingelman-Sundberg M., and Dianzani M. U. (1991) Role of ethanol-inducible cytochrome P450 (P450IIE1) in catalysing the free radical activation of aliphatic alcohols. *Biochem. Pharmacol.* **41,** 1895–1902.

Bignami A. and Dahl D. (1974) Astrocyte-specific protein and radial glia in the cerebral cortex of newborn rat. *Nature* **252,** 55–56.

Bonthius D. J. and West J. R. (1991) Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. *Teratology* **44,** 147–163.

Brenner M. (1994) Structure and transcriptional regulation of the GFAP gene. *Brain Pathol.* **4,** 245–257.

Cameron R. S. and Rakic P. (1991) Glial cell lineage in the cerebral cortex: a review and synthesis. *Glia* **4,** 124–137.

Cammer W., Tansey F., Abramovitz M., Ishigaki A., and Listowsky I. (1989) Differential localization of glutathione-S-transferase Yp and Yb subunits in oligodendrocytes and astrocytes of rat brain. *J. Neurochem.* **52,** 876–883.

Chiappelli F., Taylor A. N., Espinosa de los Monteros A., and de Vellis J. (1991) Fetal alcohol delays the development expression of myelin basic protein and transferrin in rat primary oligodendrocyte cultures. *Int. J. Dev. Neurosci.* **9,** 67–75.

Clarren S. K., Alvord E. C., Sumi S. M., Streissguth A. P., and Smith D. W. (1978) Brain malformations related to prenatal exposure to ethanol. *J. Pediatr.* **92,** 64–67.

Clarren S. K. and Smith D. W. (1978) The fetal alcohol syndrome. *N. Engl. J. Med.* **298,** 1063–1067.

Culican S. M., Baumrind N. L., Yamamoto M., and Pearlman A. L. (1990) Cortical radial glia: identification in tissue culture and evidence for their transformation to astrocytes. *J. Neurosci.* **10,** 684–692.

Davies D. L. and Cox W. E. (1991) Delayed growth and maturation of astrocytic cultures following exposure to ethanol: electron microscopic observation. *Brain Res.* **547,** 53–61.

Davies D. L. and Vernadakis A. (1984) Effects of ethanol on cultured glial cells: proliferation and glutamine synthetase activity. *Dev. Brain Res.* **16,** 27–35.

Druse M. J. (1992) Effects of in utero ethanol exposure on the development of neurotransmitter system, in *Development of the Central Nervous System: Effects of Alcohol and Opiates* (Watson R. R., ed.) Wiley-Liss, New York, pp. 139–167.

Eden S. and Cedar H. (1994) Role of DNA methylation in the regulation of transcription. *Curr. Opin. Genet. Devel.* **4,** 225–259.

Eng L. F., Vanderhaeghen J. J. Bignami A., and Gerstl B. (1971) An acidic protein isolated from fibrous astrocytes. *Brain Res.* **28,** 351–354.

Eng F. L. and Ghirnikar R. S. (1994) Glial fibrillary acidic protein and astrogliosis. *Brain Pathol.* **4,** 229–237.

Esquifino A. I., Sanchis R., and Guerri C. (1986) Effect of prenatal alcohol exposure on sexual maturation of female rat offspring. *Neuroendocrinology* **44,** 483–487.

Fein G., Meyerhoff D. J., Di Sclafani V., Ezekiel F., Poole N., MacKay S., Dillon W. P., Constans J-M., and Weiner M. W. (1994) 1H magnetic resonance spectroscopic imaging separates neuronal from glial changes in alcohol-related brain atrophy, in: *Alcohol and Glial Cells* (Lancaster F. E. ed.) National Institute on Alcohol Abuse and Alcoholism Research, Monograph 27. NIH Pub. No. 94-3742. National Institutes of Health, Bethesda, MD, pp. 227–241.

Fletcher T. L. and Shain W. (1993) Ethanol-induced changes in astrocyte gene expression during rat central nervous system development. *Alcoholism: Clin. Exp. Res.* **17,** 993–1001.

Fletcher T. L., Ingraham C. A., and Morihisa J. M. (1994) Alcohol-induced changes in astrocyte gene expression during central nervous system development: methods and mechanisms, in: Alcohol and Glial Cells (Lancaster, F. E., ed.) National Institute on Alcohol Abuse and Alcoholism, Monograph 27. NIH Pub. No. 94-3742. Bethesda, MD, pp. 103–116.

Franke H. (1995) Influence of chronic alcohol treatment on the GFAP-immunoreactivity in astrocytes of the hippocampus in rats. *Acta Histochem.* **97,** 263–271.

Fuchs E. and Weber K., (1994) Intermediate filaments: structure, dynamics, function and disease. *Ann. Rev. Biochem.* **63,** 345–382.

Furukawa S., Furukawa Y., Satoyoshi E., and Hayashi K. (1986) Synthesis/secretion of nerve growth factor is associated with cell growth in cultured mouse astroglial cells. *Biochem. Biophys. Res. Commun.* **142,** 395–402.

Garro A. J., McBeth D. L., Lima V., and Lieber C. S. (1991) Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol: Clin. Exp. Res.* **15,** 395–398.

Goodlett C. R., Leo J. T., O'Callaghan J. P., Mahoney J. C., and West J. R. (1993) Transient cortical astrogliogenesis induced by alcohol exposure during the neonatal brain growth spurt in rats. *Dev. Brain Res.* **72,** 85–97.

Gressens P., Lammens M., Picard J. J., and Evrard, P. (1992) Ethanol-induced disturbances of gliogenesis and neurogenesis in the developing murine brain: an in vitro and in vivo immunohistochemical and ultrastructural study. *Alcohol Alcoholism* **27,** 219–226.

Guerri C., Sáez R., Portolés M., and Renau-Piqueras J. (1993) Derangement of astrogliogenesis as a possible mechanism involved in alcohol-induced alterations of central nervous system development. *Alcohol Alcoholism* **(suppl. 2),** 203–208.

Guerri C., Esquifino A., Sanchis R., and Grisolía S. (1984) Growth, enzymes and hormonal changes in offspring of alcohol-fed rats, in *Mechanisms of Alcohol Damage in Utero.* Ciba Foundation, Pitman, London, pp. 85–102.

Guerri C., Sáez R., Sancho-Tello M., Martin de Aguilera M., and Renau-Piqueras J. (1990) Ethanol alters astrocyte development: a study of critical periods using primary cultures. *Neurochem. Res.* **15,** 559–565.

Guerri C., Montoliu C., and Renau-Piqueras J. (1994) Involvement of free radical mechanism in the toxic effects of alcohol: implications for fetal alcohol syndrome, in: *Free Radicals in Diagnostic Medicine* (Armstrong, D. ed.) Plenum, New York, pp. 291–305.

Hansson E. and Rönnbäck L. (1995) Astrocytes in glutamate neurotransmission. *FASEB J.* **9,** 343–350.

Hatten M. E., Liem R. K. H., Shelanski, M. L., and Mason C. A. (1991) Astroglia in CNS injury. *Glia* **4,** 233–243.

Hatten M. E. (1990) Riding the glial monorail: a common mechanism for glial-guided migration in different regions of the development brain. *Trends Neurosci.* **13,** 179–184.

Heaton M. B., Swanson D. J., Paiva M., and Walker D. W. (1992) Ethanol exposure affects trophic factor activity and responsiveness in chick embryo. *Alcohol* **9,** 161–166.

Iborra F. J., Renau-Piqueras J., Portolés M., Boleda M. D., Guerri C., and Pares X. (1992) Immunocytochemical and biochemical demonstration of formaldehyde dehydrogenase (class III alcohol dehydrogenase) in the nucleus. *J. Histochem. Cytochem.* **40,** 1865–1878.

Inagaki M., Nakamura Y., Takeda M., Nishimura T., and Inagaki N. (1994) Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation. *Brain Pathol.* **4,** 239–243.

Ingelman-Sundberg M. (1993) Ethanol-inducible cytochrome P4502E1. Regulation, radical formation and toxicological importance, in *Free Radicals: From Basic Science to Medicine* (Poli G.,

Albano E., and Dianzani M. U. eds.) Birkhäuser Verlag, Basel. pp. 287–301.

Kaneko R., Hagiwara N., Leader K., and Sueoka N. (1994) Glial-specific cAMP response of the glial fibrillary acidic protein gene in the RT4 cell lines. *Proc. Natl. Acad. Sci. USA* **91,** 4529–4533.

Kennedy L. A. and Mukerji S. (1986) Ethanol neurotoxicity. I. Direct effects on replicating astrocytes. *Neurobehav. Toxicol. Teratol.* **8,** 11–21.

Kentroti S. and Vernadakis A. (1997) Differential expression in glial cells derived from chick embryo cerebral hemispheres at an advanced stage of development. *J. Neurosci. Res.* **47,** 322–331.

Kimelberg H. K. and Aschner M. (1994) Astrocytes and their functions, past and present, in *Alcohol and Glial Cells* (Lancaster F. E., ed.) National Institute on Alcohol Abuse and Alcoholism Research, Monograph 27. NIH Pub. No. 94-3742. Bethesda, MD, pp. 1–40.

Klassen L. W., Tuma D., and Sorell M. F. (1995) Immuno mechanisms of alcohol-induced liver disease. *Hepatology* **22,** 355–357.

Kotch L. E., Chen S-Y., and Sulik, K. K. (1995) Ethanol-induced teratogenesis: free radical damage as a possible mechanism. *Teratology* **52,** 128–136.

Lancaster F. E. (ed.) (1994) *Alcohol and Glial Cells.* National Institute on Alcohol Abuse and Alcoholism Research, Monograph 27. NIH Pub. No. 94-3742, National Institutes of Health, Bethesda, Maryland.

Laping N. J., Teter B., Nichols N. R., Rozovsky I., and Finch C. E. (1994) Glial bibrillary acidic protein: regulation by hormones, cytokines, and growth factors. *Brain Pathol.* **1,** 259–275.

Ledig M. and Tholey G. (1994) Fetal alcohol exposure and glial cells development, in *Alcohol and Glial Cells* (Lancaster, F. E., ed.) National Institute on Alcohol Abuse and Alcoholism Research, Monograph 27. NIH Pub. No. 94-3742. National Institutes of Health, Bethesda, MD, pp. 117–132.

Liedtke W., Edelman W., Bieri P. L., Chiu C., Cowan N. J., Kucherlapati R., and Raine C. S. (1996) GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. *Neuron* **17,** 607–615.

Lieth E., Towle A. C., and Lauder J. M. (1989) Neuronal-glial interactions: quantitation of astrocytic influences on development of catecholamine neurons. *Neurochem. Res.* **14,** 979–985.

Lokhorst D. K. and Druse M. J. (1993) Effects of ethanol on cultures fetal astroglia. *Alcoholism: Clin. Exp. Res.* **17,** 810–815.

Luo J. and Miller M. W. (1996) Ethanol inhibits basic fibroblast growth factor-mediated proliferation of C6 astrocytoma cells. *J. Neurochem.* **67,** 1448–1456.

Makar T. K., Nedergaard M., Preuss A., Gelbard A. S., Perumal A. S., and Cooper A. J. L. (1994) Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. *J. Neurochem.* **62,** 45–53.

Mayordomo F., Renau-Piqueras J., Megias L., Guerri C., Iborra F. J., Azorin I., and Ledig M. (1992) Cytochemical and stereological analysis of rat cortical astrocytes during development in primary culture. Effect of prenatal exposure to ethanol. *Int. J. Dev. Biol.* **36,** 311–321.

Mikami K., Haseba T., and Ohno Y. (1997) Ethanol induces transient arrest of cell division (G2+M block) followed by G0/G1 block: dose effects of short- and longer-term ethanol exposure on cell cycle and cell functions. *Alcohol Alcoholism* **32,** 145–152.

Miller M. W. (1992) The effects of prenatal exposure to ethanol on cell proliferation and neuronal migration, in *Developmental of the Central Nervous System: Effects of Alcohol and Opiates* (Miller M., ed.) Liss, New York, pp. 47–69.

Miller M. W. and Robertson S. (1993) Prenatal exposure to ethanol alters the postnatal development and transformation of radial glia to astrocytes in the cortex. *J. Comp. Neurol.* **337,** 253–266.

Montoliu C., Vallés S., Renau-Piqueras J., and Guerri C. (1994) Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. *J. Neurochem.* **63,** 1855–1862.

Montoliu C., Sancho-Tello M., Azorin I., Burgal M., Vallés S., Renau-Piqueras J., and Guerri C. (1995) Ethanol increases cytochrome P4502E1 and induces oxidative stress in astrocytes. *J. Neurochem.* **65,** 2561–2570.

Müller H. S., Junghans U., and Kappler J. (1994) Astroglial neurotrophic and neurite-promoting factors. *Pharmacol. Ther.* **65,** 1–18.

Nordman R., Ribière C., and Rouach H. (1992) Implication of free radical mechanisms in ethanol-induced cellular injury. *Free Radical Biol. Med.* **12,** 219–225.

Norenberg M. D., Hertz L., and Schousboe A., eds. (1988) *The Biochemical Pathology of Astrocytes*, Liss, New York.

Norris C. R. and Kalil K. (1991) Guidance of callosal axons by radial glia in the developing cerebral cortex. *J. Neurosci.* **11,** 3481–3492.

Orrenius S. (1993) Mechanism of oxidative cell damage, in *Free Radicals: From Basic Sciences to Medicine* (Poli G., Albano E., and Dianzani M. U., eds.) Birkhäuser Verlag, Basel, pp. 47–64.

Peiffer J., Majewski F., Fischbach H., Bierich J. R., and Volk B. (1979) Alcohol embryo- and fetopathy. *J. Neurol. Sci.* **41,** 125–137.

Pennington S. (1988) Ethanol-induced growth inhibition: the role of cyclic AMP-dependent protein kinase. *Alcoholism: Clin. Exp. Res.* **12,** 125–130.

Pérez-Torrero E., Durán P. Granados L., Gutiérrez-Ospina G., Cintra L., and Díaz-Cintra S. (1997) Effects of acute prenatal ethanol exposure on Bergmann glia cells early postnatal development. *Brain Res.* **746,** 305–308.

Phillips D. E. (1992) Effects of alcohol on the development of glial cells and myelin, in *Alcohol and Neurobiology: Brain Development and Hormone regulation* (Watson RR, ed.) CRC Press, Boca Raton, FL, pp. 83–108.

Phillips D. E. (1994) Effects of alcohol on glial cell development. In vivo: morphological studies, in: *Alcohol and Glial Cells* (Lancaster, F. E., ed.) National Institute on Alcohol Abuse and Alcoholism Research, Monograph 27. NIH Pub. No. 94-3742. Bethesda, MD, pp. 69–91.

Pinazo-Duran M. D., Renau-Piqueras J., and Guerri C. (1993) Developmental changes in the optic nerve related to ethanol consumption in pregnant rats: analysis of the ethanol-exposed optic nerve. *Teratology* **48,** 305–322.

Popova E. N. and Shchekalina G. A. (1980) Effect of alcohol on glial cells. *Z. Neuropatol. Psikhiatr.* **80,** 539–544.

Portolés M., Sanchis R., and Guerri C. (1988) Thyroid hormone levels in rats exposed to alcohol during development. *Horm. Met. Res.* **20,** 267–270.

Rakic P. (1991) Glial cells in development. In vivo and in vitro approaches. *Ann. NY Acad. Sci.* **633,** 96–99.

Ramón y Cajal S. (1911) Histologie du système nerveux de l'homme et des vertébrés. Malone, Paris.

Raps S. P., Lai J. C. K., Hertz L., and Cooper A. J. L. (1989) Glutathione is present in high concentrations in cultured astrocytes but nor in cultured neurons. *Brain Res.* **493,** 398–401.

Razin A. and Shemer R. (1995) DNA methylation in early development. *Hum. Mol. Gen.* **4,** 1751–1755.

Reed D. J. (1990) Glutathione: toxicological implications. *Ann. Rev. Pharmacol. Toxicol.* **30,** 2395–3407.

Reichenbach A. (1989) Glia: neuron index: review and hypothesis to account for different values in various mammals. *Glia* **2,** 71–77.

Renau-Piqueras J., Zaragoza R., De Paz P., Báguena-Cervellera R., Megias L., and Guerri C. (1989) Effects of prolonged ethanol exposure on the glial fibrillary acidic protein-containing intermediate filaments of astrocytes in primary culture: a quantitative immunofluorescence and immunogold electron microscopic study. *J. Histochem. Cytochem.* **37,** 229–240.

Renau-Piqueras J., Guasch R., Azorin I., Seguí J. M., and Guerri C. (1997) Prenatal alcohol exposure affects galactosyltransferase activity and glycoconjugates in the Golgi apparatus of fetal rat hepatocytes. *Hepatology* **25,** 343–350.

Renau-Piqueras J., Guerri C., Burgal M., De Paz P., Sáez R., and Mayordomo F. (1992) Prenatal exposure to ethanol alters plasma membrane glycoproteins of astrocytes during development in primary culture as revealed by concanavalin A binding and 5-nucleotidase activity. *Glia* **5,** 65–74.

Riley E. P., Mattson S. N., Sowell E. R., Jernigan T. L., Sobel D. F., and Jones K. L. (1995) Abnormalities of the corpus callosum in children prenatally exposed to alcohol. *Alcoholism: Clin. Exp. Res.* **19,** 1198–1202.

Riol H., Fafes C., and Tardy M. (1992) Transcriptional regulation of glial fibrillary acidic protein (GFAP)-mRNA expression during postnatal development of mouse brain. *J. Neurosci. Res.* **32,** 79–85.

Sáez R., Burgal M., Renau-Piqueras J., Marqués A., and Guerri C. (1991) Evolution of several cytoskeletal proteins of astrocytes in primary culture: effect of prenatal alcohol exposure. *Neurochem. Res.* **16,** 737–747.

Sagara J., Miura K., and Bannai S. (1993) Maintenance of neuronal glutathione by glial cells. *J. Neurochem.* **61,** 1667–1676.

Sanchis R., Sancho-Tello M., and Guerri C. (1986) The effects of chronic ethanol alcohol consumption on pregnant rats and their offspring. *Alcohol Alcoholism* **21,** 295–305.

Sancho-Tello M., Vallés S., Montoliu C., Renau-Piqueras J., and Guerri C. (1995) Developmental

pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. *Glia* **15,** 157–166.

Shafit-Zagardo B., Kume-Iwaki A., and Goldman J. E. (1988) Astrocytes regulate GFAP mRNA levels by cyclic AMP- and protein kinase C-dependent mechanisms. *Glia* **1,** 346–354.

Shetty A. K. and Phillips D. E. (1992) Effects of prenatal ethanol exposure on the development of Bergmann glia and astrocytes in the rat cerebellum: an immunohistochemical study. *J. Comp. Neurol* **321,** 19–32.

Silver J. and Ogawa M. Y. (1983) Postnatally induced formation of the corpus callosum in acallosal mice on glia-coated cellulose bridges. *Science* **220,** 1067–1069.

Slater S. J., Cox K. J., Lombardi J. V., Ho C., Kelly M. B., Rubin D., and Stubbs C. D. (1993) Inhibition of protein kinase C by alcohols and anaesthetic. *Nature* **364,** 82–84.

Smith D. E. and Davies D. L. (1990) Effect of perinatal administration of ethanol on the C1 pyramidal cell of the hippocampus in purkinje cell of the cerebellum: an ultrastructural survey. *J. Neurocytol.* **19,** 708–712.

Snyder A. K., Singh S. P., and Ehmann S. (1994) Effects of ethanol on substrate uptake and incorporation in primary astrocyte cultures, in: *Alcohol and Glial Cells* (Lancaster, F. E., ed.) National Institute on Alcohol Abuse and Alcoholism, Monograph 27. NIH Pub. No. 94-3742. Bethesda, MD, pp. 93–102.

Streissguth A. P., Sampson P. D., Olson H. C., Bookstein F. L., Barr H. M., Scott M., Feldman J., and Mirsky A. F. (1994) Maternal drinking during pregnancy: attention and short-term memory in 14-years-old offspring a longitudinal prospective study. *Alcoholism: Clin. Exp. Res.* **18,** 202–218.

Swayze V. W., Johnson V. P., Hanson J. W., Piven J., Sato Y., Giedd J. N., Mosnik D., and Andreasen N. C. (1997) Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. *Pediatrics* **99,** 232–240.

Tavares M. A. and Paula-Brabosa M. M. (1984) Remodeling of the cerebellar glomeruli after long-term alcohol consumption in the adult rat. *Brain Res.* **309,** 217–226.

Tindberg N. and Ingelman-Sundberg, M. (1996) Expression, catalytic activity, and inducibility of cytochrome P450 2E1 (CYP2E1) in the rat central nervous system. *J. Neurochem.* **67,** 2066–2073.

Vallés S., Lindo L., Montoliu C., Renau-Piqueras J., and Guerri C. (1994) Prenatal exposure to ethanol induces changes in the nerve growth factor and its receptor in proliferating astrocytes in primary culture. *Brain Res.* **656,** 281–286.

Vallés S., Pitarch J., Renau-Piqueras J., and Guerri C. (1997) Ethanol exposure affects GFAP gene expression and transcription during rat brain development. *J. Neuro chem.*, in press.

Vallés S., Sancho-Tello M., Miñana R., Climent E., Renau-Piqueras J., and Guerri C. (1996) Glial fibrillary acidic protein expression in rat brain and in radial glia culture is delayed by prenatal ethanol exposure. *J. Neurochem.* **67,** 2425–2433.

Weinberg J. (1994) Recent studies on the effects of fetal alcohol exposure on the endocrine and immune systems. *Alcohol Alcoholism* **2,** 401–409.

Weinstein D. E., Shelanski M. L., and Liem R. K. H. (1991) Suppression by antisense mRNA demonstrates a requirement for the glial fibrillary acidic protein in the formation of stable astrocytic processes in response to neurons. *J. Cell Biol.* **112,** 1205–1213.

Whitaker-Azmitia P. M. and Azmitia E. C. (1989) Stimulation of astroglial serotonin receptors produces culture media which regulates growth of serotonergic neurons. *Brain Res.* **497,** 80–85.

Whitaker-Azmitia P. M., Druse M., Walker P., and Lauder J. M. (1996) Serotonin as a developmental signal. *Behav. Brain Res.* **73,** 19–29.