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Chapter 26

Fetal alcohol spectrum disorder: pathogenesis and mechanisms

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INTRODUCTION

With over four decades of clinical investigation and 100 years of basic research, much has been learned about the birth defects that result from prenatal alcohol (ethanol) exposure in people and animal models. This is especially true for congenital abnormalities involving the nervous system. Our current understanding of the cascades of altered developmental processes that occur subsequent to an initial interaction of alcohol and/or its metabolites with an endogenous molecule and that contribute to structural and functional birth defects (i.e., alcohol's teratopathogenesis) is substantial and growing. In this chapter, no distinction is made between the impact of alcohol and its major metabolite, acetaldehyde, which is also teratogenic. Among the known major components of the cascades of alcohol-induced developmental damage are excessive cell death, changes in the cell cycle and proliferation, cell migration, cell morphogenesis, and gene expression, as well as free radical damage and interference with cell signaling. For these, upstream and downstream events are described as they are considered pertinent and informative. While the molecular events that initiate alcohol's teratopathogenesis (i.e., its primary mechanisms) remain for the most part unidentified, a synopsis of emerging mechanistic data is presented.

As reflected in the term, fetal alcohol spectrum disorder (FASD), prenatal alcohol exposure can result in myriad abnormal endpoints. While the emphasis in this chapter is on alcohol-induced neuroteratogenesis, nonneural tissues and organs, including facial and ocular structures, the heart, and limbs, are also frequently involved as part of FASD. Alcohol-induced birth defects involving one or more structures or systems may result from one predominant primary mechanism and/or pathogenic cascade or from simultaneous insult to more than one distinct pathway. This, along with the fact that an initial insult to a selected cell population is expected to subsequently adversely affect other cell populations and/or developmental events, commonly makes interpretation of experimental data complex.

As for other teratogens, alcohol's adverse prenatal effects are developmental stage-dependent, with many critical periods having been identified. Study of animal models has shown that a variety of different structural abnormalities, including those of the brain, can result from alcohol insult limited to specific times during the embryonic period. Exemplary are studies of a mouse FASD model in which differing patterns of brain and facial dysmorphology result from acute maternal alcohol exposure on gestational day (GD) 7 versus 8.5 (Lipinski et al., 2012), and in which heart, kidney, and limb defects follow alcohol insult on GDs 8.5, 9, and 9.25, respectively (Daft et al., 1986; Gage and Sulik, 1991; Kotch et al., 1992). Developmental stages present in mice on GDs 7 through 9 correspond to those in humans during the third through fourth weeks after fertilization. Additionally, alcohol exposure limited to even narrow windows of time during early embryogenesis or involving any or all of the three trimester equivalents can yield functional (including behavioral) defects.

In addition to developmental stage dependency, the type and severity of alcohol-induced abnormality are dependent upon exposure pattern and dosage, genetic background, and other environmental influences, including maternal nutrition, stress, and other coadministered drugs. Research employing model systems has facilitated definition of these modulating factors and has provided a wealth of information regarding alcohol's effects on both the mother and the conceptus (reviewed by Goodlett and Horn, 2001; Chen et al., 2003; Cudd,

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2005; Miller, 2006; Schneider et al., 2011). The focus of this chapter is on alcohol-induced damage to the developing baby, itself, providing an overview of exemplary data rather than an exhaustive literature review.

CELL DEATH

Cell death is an event common to the teratogenic process initiated by many agents. Early reports of prenatal alcohol exposure and teratogenesis in model systems describe cell degeneration (Sandor et al., 1980; Bannigan and Burke, 1982; Bannigan and Cottell, 1984), as do more recent basic FASD studies, the latter of which include distinction between two types of alcohol-induced cell death – necrosis and apoptosis (Smith, 1997). Necrosis is characterized by swelling of cytoplasmic organelles, rupture of the plasma membrane with uncontrolled release of cellular components, and subsequent local inflammation. Typically, alcohol-induced necrosis involving cells of the nervous system is produced by a relatively fulminant insult or following alcohol withdrawal. While necrosis is traditionally viewed as a non-regulated form of cell death, it is now recognized that some necrotic cell death may be executed through a signaling pathway-dependent mechanism termed necroptosis or programmed necrosis, a process that has been implicated in mediating neuronal excitotoxicity (Christofferson and Yuan, 2010).

In the developing brain, apoptosis, a controlled form of cellular self-destruction, appears to be the most typical form of alcohol-induced cell death. Following alcohol-mediated modulation of extracellular or intracellular signals the apoptotic cascade is triggered, with subsequent release of mitochondrial proteins and cytochrome c and activation of cysteine proteases called caspases. Apoptosis involves nuclear condensation (pyknosis), DNA fragmentation, and the formation of membrane-bound cellular fragments termed apoptotic bodies. Excessive apoptotic cell death has been identified in neuronal as well as non-neuronal cell types in FASD models (Cartwright et al., 1998; Dunty et al., 2001; Olney et al., 2002a, b; Johnson et al., 2007). Demonstrating the significant role of apoptosis in alcohol teratogenesis are studies showing the antiteratogenic effect of preventing alcohol-induced reduction in the antiapoptotic Bcl2 protein (Sari et al., 2012), bolstering Bcl2 levels genetically (Heaton et al., 1999) or pharmacologically (Ullah et al., 2012), and treatment with the caspase inhibitor, CED-3 protease (Cartwright et al., 1998).

FASD research conducted primarily in rodents has highlighted the first- and third-trimester equivalents as times when excessive cell death plays a major role in alcohol-induced neuroteratogenesis. Findings from both exposure periods illustrate that the excessive cell death is not uniformly distributed, but is temporally, regionally, and cell type-specific. With an eye toward defining the involved pathogenetic cascade(s), studies employing acute alcohol exposure paradigms and identification of cell death patterns present within hours following treatment have been particularly informative.

With respect to acute exposures occurring during the first trimester, vital staining of mouse embryos within 12 hours of maternal alcohol treatment has allowed a relatively comprehensive temporospatial determination of cell populations that are killed both within and outside the CNS (Sulik et al., 1988; Kotch et al., 1992; Kotch and Sulik, 1992; Dunty et al., 2001, 2002). As illustrated in Figure 26.1, relatively large proportions of the cells in selected regions of the developing forebrain and hindbrain are vulnerable at early stages of development to alcohol-induced cell death. Included are the cells at the rim of the forebrain neural folds; cells that upon closure of the anterior neuropore normally form midline tissues. In the hindbrain the alar plate as well as premigratory neural crest cells are vulnerable. In addition to Nile blue sulfate uptake, TUNEL assays confirm the cell death to be apoptotic. Comparable findings in studies employing a chick embryo model (Cartwright and Smith, 1995a, b; Cartwright et al., 1998) have been followed by experiments showing that teratogenic alcohol levels rapidly elicit a G protein-stimulated rise in phospholipase C activity and an intracellular Ca^{2+} transit (Garic-Stankovic et al., 2005). Recent studies have shown that downstream of the alcohol-induced calcium transient is repression of the transcriptional effector, β-catenin. Blockade of the calcium transient normalizes β-catenin activity and prevents cell death (Flentke et al., 2011).

The third trimester is a time when differentiating neurons are sending out axons and making synaptic connections and when approximately one-third of the postmitotic neuronal population undergoes naturally occurring apoptotic cell death, thus eliminating surplus cells. Cell death induced by alcohol during this time period has been most studied in neonatal (third-trimester equivalent) rodents (Olney et al., 2002a; Young and Olney, 2006; Idrus and Napper, 2012). The results of this work suggest that the alcohol-induced cell death represents an abnormal augmentation of the normal cell death process, a premise upon which hypotheses regarding a role for alcohol-mediated alterations in the function (e.g., via changes in receptor activity) or abundance of neuronal survival factors are largely based. Implicated are alterations in nerve growth factor, brain-derived neurotrophic factor (BDNF), glia-derived neurotrophic factor, insulin-like growth factor-1 (IGF-1), heparin-binding epidermal growth factor *N*-methyl-D-aspartate (NMDA), and pituitary adenylyl cyclase activating polypeptide (Kilburn et al., 2006; Miller, 2006).

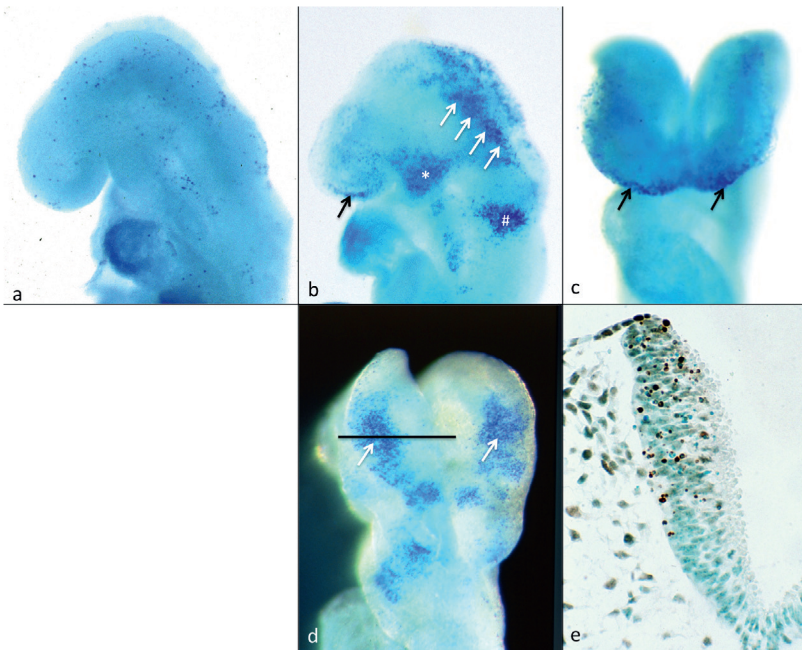

Fig. 26.1. Vital staining with Nile blue sulfate (NBS) in control (**a**) and alcohol-exposed mouse embryos (lateral view in **b**, frontal view in **c**, and dorsal view in **d**) illustrates excessive stain uptake (dark blue stipple), as is indicative of apoptotic cell death, in the latter. The NBS staining pattern illustrates excessive cell death in selected cell populations, including those in the rim of the forebrain neural folds (black arrows), the first branchial arch (*), the otic placode (#), and the alar plate of the hindbrain (white arrows). TUNEL labeling of a histologic section through the hindbrain (**E**) confirms apoptotic cell death in the alar versus the basal plate. (Modified from Dunty et al., 2001, and Kotch and Sulik, 1992.)

As reviewed by Goodlett et al. (2005), studies employing animal models have shown that, during the third-trimester equivalent period of synaptogenesis, postmitotic neurons in the cerebral cortex, hypothalamus, cerebellum, and brainstem are killed by alcohol. For the neurons in these various brain regions, temporal windows of vulnerability vary, as exemplified by results of acute (binge) exposure-based experiments showing that maximal numbers of Purkinje neurons are killed approximately 3 days earlier than are cerebrocortical neurons. While large alcohol doses have been employed in most of these studies, Young and Olney (2006) have reported that, in infant mice, a rise in blood alcohol concentration to a moderate level of approximately 50 mg/dL for a duration of 30–45 minutes is sufficient to cause widespread neuroapoptosis. Further research (Farber et al., 2010) has also included examination of alcohol-induced neuroapoptosis in fetal macaque brains following high-dose, 8-hour maternal alcohol treatment at various times between days 105 and 155 of their 160–165-day gestation period. A 60-fold increase in apoptosis above control levels was found in numerous fetal brain regions, with patterns of effect being consistent with those in rodents.

Study of alcohol-induced apoptotic cell death as occurs during the period of synaptogenesis has shown that, while patterns of caspase 3 activation and cell death are corresponding (Olney et al., 2002a), caspase 3 knockout mice remain vulnerable to alcohol-induced neuronal death (Young et al., 2005). Thus, caspase 3 does not appear to be an essential factor in determining whether cell death will occur. Indeed, a caspase-3-independent cell death pathway that is related to Bax translocation into mitochondria followed by the mitochondrial permeability transition and release of death-promoting factors including Smac/DIABLO, apoptosis-inducing factor and EndoG, has been reported (Miller, 1997; Stefanis et al., 1999; Chang et al., 2003). Alcohol-induced developmental neuroapoptosis does not occur in Bax knockout mice, indicating that translocation of Bax protein to mitochondria is an essential step (Young et al., 2003). It is speculated that, upstream of this event, alcohol-mediated suppression of neuronal activity via a combination of NMDA receptor blockade and hyperactivation of GABA-A receptors triggers the cell death process (Ikonomidou et al., 2000). Another upstream event that may be involved in alcohol-induced neuroapoptosis is suppression of the phosphorylation of ERK, an extracellular signal-regulated protein kinase that is believed to play a role in cell survival (Young et al., 2008). This is supported by the finding that lithium, which stimulates ERK phosphorylation, prevents the alcohol-induced phosphorylation suppression.

Studies of neuronal cell death resulting from alcohol exposure during the third-trimester equivalent have also focused on excitotoxic cell death as occurs during withdrawal and results from overactivity of NMDA receptors. Supporting a role for this effect are studies of neonatal rats in which NMDA receptor antagonists have been shown to effectively attenuate alcohol-induced abnormalities, including motor and learning deficits and reductions in Purkinje cell numbers (Thomas et al., 2002; Idrus et al., 2011).

CELL CYCLE AND PROLIFERATION

Animal model-based studies of alcohol-induced cell cycle and proliferation alterations have focused, to a large extent, on insult occurring during the second-trimester equivalent, when most neurogenesis occurs. In rats, a species in which cortical neurogenesis begins on GD 11 and ends on GD 21 (comparable to weeks 6 through 17 of human development), prenatal alcohol exposure delays and extends the period of neuronal generation by 1 day (Miller, 1986, 2006). Notably, increases in the time spent by neurons in the G1 phase of the cell cycle following prenatal alcohol exposure appears to result from interference with growth factors. Supporting studies show that IGF receptor function/signaling is reduced in the presence of alcohol (Resnicoff et al., 1993) and that alcohol prevents tyrosine autophosphorylation of type 1 IGF receptors, and inhibits cyclins and cyclin-dependent kinases (Li et al., 2002).

Of interest, a computational model indicates that cell cycle perturbations occurring in rodent models during the second-trimester equivalent have a more significant effect than synaptogenesis-related insult occurring during the third-trimester equivalent (Gohlke et al., 2005). Additionally, these investigators suggest that the developing human neocortex may be more sensitive to the effects of alcohol than the rodent as a result of the relative increase in the length of neurogenesis and subsequent increased neocortical size in humans. They also predict that neuronal death during synaptogenesis may play a greater role in shaping the brain in primates than in rodents.

CELL MIGRATION

In animal models, alcohol has been shown to reduce the rate of neuronal migration during the inside-out development of the cortex and to cause migrating neurons to finish their migration in inappropriate locations. This is evidenced by studies showing that, following prenatal alcohol exposure from GD 6 to term in rats (comparable to weeks 3 through 18 of human development), many of the neurons that are generated late in gestation and that should populate cerebral cortical layer II/III instead either terminate their migrations in layers IV, V, or VI or overmigrate into layer I (Miller, 1986, 1993). Speculation regarding likely mechanisms involved in alcohol-induced neuronal migration errors includes disruption of cell adhesion, specifically involving L1 cell adhesion molecule, and interference with glial-guided migration or detachment of neurons from glial fibers. Notably, alcohol causes premature transformation of radial glia into astrocytes, with associated migrating neurons being unable to complete their migration (Miller and Robertson, 1993). Also implicated in neuronal migrational errors are effects on cytoskeletal elements, integrins, extracellular matrix components, and growth factors (Miller, 2006).

In addition to migration error-based intracortical defects, supracortical abnormalities presenting as suprapial/leptomeningeal heterotopias result from prenatal alcohol exposure. Results of *in vivo* and *in vitro* studies of fetal rat cortices suggest that these defects result from alcohol-induced alterations in the pial membrane and glia limitans (Komatsu et al., 2001; Mooney et al., 2004). In turn, these alterations are hypothesized to be secondary to an initial interference with growth factor regulation, leading to reduced expression of reelin and subsequent aberrant movement of neurons into the cortical marginal zone and beyond (Mooney et al., 2004). A recent study of mice in which prenatal alcohol exposure was limited to just GD 7 (comparable to the mid third week of human development), a time when the neural plate is just beginning to form, has shown that even at this very early stage of development alcohol can cause severe cortical migration errors, including leptomeningeal heterotopias (Fig. 26.2; Godin et al., 2010). These results suggest that alcohol damages or kills the progenitors of cells that are key to cerebrocortical cell migration. Recognizing that, on GD 7 in mice, the developing septal region is particularly vulnerable and that this brain region is an important source of reelin-producing Cajal-Retzius cells, it is hypothesized that adverse effects on this cell population underlie the cerebrocortical migration errors that follow this early insult.

CELL MORPHOGENESIS

Both *in vivo* and *in vitro* animal studies have shown that prenatal alcohol exposure affects dendritic morphology. Exemplary are the results of examination of the apical dendrite of hippocampal CA1 pyramidal cells from postnatal rats whose mothers had been chronically alcohol-exposed prior to and throughout pregnancy and nursing (Tarelo-Acuna et al., 2000). In the alcohol-exposed dendrites a relatively high proportion of stubby or wide spines were found, a change that is expected to affect electrical excitability and firing pattern. Cui

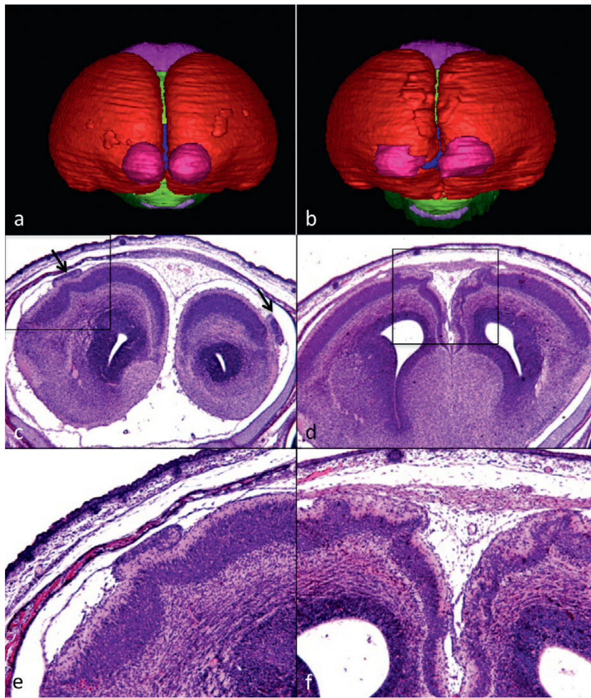

Fig. 26.2. Magnetic resonance imaging-based brain reconstructions (**a, b**) and histologic sections (**c–f**) of alcohol-affected fetal mice that had been acutely treated with alcohol on their 7th gestational day (comparable to mid third week of human development) illustrate cerebrocortical migration errors. In the specimen shown in **a, c,** and **e** the defects are leptomeningeal heterotopias (arrows in **c**) while cortical dysplasia is present in the specimen in **b, d,** and **f**. Boxed areas in **c** and **d** are shown at higher magnification in **e** and **f**, respectively. Color codes in **a** and **b** are as follows: cerebral cortex, red; olfactory bulbs, pink; mesencephalon, magenta; diencephalon, green; septal region, blue; pituitary, purple. (Modified from Godin et al., 2010.)

et al. (2010) also report dendritic spine changes in their study of pyramidal cells in the visual cortex of postnatal mice following chronic prenatal (first- and second-trimester equivalent) alcohol exposure. While this study showed decreased numbers of spines, their mean length was increased. Additionally noted were changes in synaptic ultrastructure. Histologic analyses also reveal alcohol-induced changes in overall dendritic form. For example, in the shell of the nucleus accumbens of adult rats that had been chronically alcohol-exposed during their gestation, the dendrites of medium spiny neurons were found to be reduced in length and to be less branched than in controls; results that are suggestive of effects on reward processing (Rice et al., 2012). In keeping with the *in vivo* studies, cultured rat hippocampal pyramidal neurons exhibit alcohol-induced correlative decreases in total dendritic length and synapse number (Yanni and Lindsley, 2000). Other *in vitro* studies of this cell type have shown that alcohol induces alterations in axonal growth dynamics and have provided evidence suggesting that this is a result of disruption of neurotrophic factor regulation (Lindsley et al., 2011).

In addition to *in vitro* studies of hippocampal pyramidal neurons, cerebellar granule cells cultured in the presence of alcohol have been examined. In these neurons, L1 cell adhesion molecule (L1) has a neurite outgrowth-promoting activity that appears to result from its activation of Src family kinases, the scaffolding protein Cas, and extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Chen and Charness, 2012). L1-mediated axon outgrowth can be inhibited by pharmacologic concentrations of alcohol, an effect that appears at least in part to involve alcohol-induced retardation of L1 trafficking through lipid rafts (Tang et al., 2011). Lending support to this premise are the results of an acute postnatal day 6 rat (comparable to 31–32 weeks of human prenatal development) *in vivo* alcohol exposure study showing a significant proportional increase of L1 in cerebellar lipid rafts (Littner et al., 2013). Importantly, the peptide, NAP (an active motif of activity dependent neuroprotective protein) which, like L1, can stimulate axon outgrown in cultured cerebellar granule cells, reduces alcohol's inhibition of L1 adhesion (Wilkemeyer et al., 2002). *In vivo* and in whole embryo culture systems this peptide, along with SAL, a short peptide from activity-dependent neurotrophic factor protein, are antiteratogenic (Spong et al., 2001; Poggi et al., 2003; Wilkemeyer et al., 2003, 2004; Chen et al., 2005; Sari and Gozes, 2006; Parnell et al., 2007; Zhou et al., 2008). Protection afforded by these peptides has been linked to prevention of an alcohol-induced rise in BDNF (Incerti et al., 2010).

Of major importance in our increasing understanding of alcohol's mechanism of action is recognition of its molecular interactions. At the forefront of this work is that related to defining the interaction between alcohol and L1. L1 homophilic binding, as occurs in L1 mediated cell-cell adhesion, is potentiated by folding of the extracellular domain of the molecule into a horseshoe structure in which the immunoglobulin 1 and 4 domains lie opposed to each other. Photolabeling experiments have illustrated that alcohol interacts with a binding pocket at the Ig1–Ig4 interface (Dou et al., 2011). Phosphorylation of the L1 cytoplasmic domain regulates the conformation and function of the extracellular domain, a phenomenon known as inside-out signaling. Recently, sensitivity of L1 to alcohol has been shown to be regulated by phosphorylation of an ERK2 substrate (S1248) on L1's intracellular domain. The relevance of this finding to FASD is supported by the fact that ERK2 is a component of the MAPK signaling pathway and that *in vitro* as well as *in vivo* studies show that

MAPK activity differences correspond to sensitivity to alcohol-induced cell adhesion inhibition and teratogenesis (Dou et al., 2013).

GENE EXPRESSION CHANGES

Genetic

New genomic methodologies, especially microarray analyses, have provided a way to screen the expression (conversion to a gene product) of large numbers of genes in order to identify alcohol-induced changes. Green et al. (2007) used this technique in comparing alcohol-induced gene expression changes in two closely related mouse substrains (C57Bl/6N and C57Bl/6J) that show differing teratogenic responses to an acute early (GD 8; comparable to early in the fourth week of human development) insult. Gene expression and pathway analyses revealed downregulation of ribosomal proteins and upregulation of glycolysis and the pentose phosphate pathway just in the C57Bl/6 N embryos and upregulation of tight junction, focal adhesion, adherens junction and actin cytoskeleton regulation pathways in both substrains. Based on gene expression changes, entry points for alcohol were identified at several hubs, including MAPK1, ALDH3A2, IGF-1, and EGFR. Overall the findings from this study support the view that developmental exposure to alcohol alters signaling pathways linking receptor activation to cytoskeletal reorganization.

A similar and more recent comparison of DBA/2J mice, which are relatively resistant to alcohol teratogenesis, and the susceptible C57Bl/6J strain showed that alcohol exposure decreased expression of many genes and increased relatively few (Downing et al., 2012). Genes found to be preferentially downregulated in the C57Bl/6J mouse strain are those involved in transcription, translation, and mRNA splicing. Additionally, genes involved in protein synthesis, the ubiquitin pathway, and epigenetic genes involved in chromatin remodeling and methylation were found to be important alcohol targets. Supporting these findings are results of whole embryo and neuronal cell culture-based microarray analyses (Gutala et al., 2004; Liu et al., 2009; Zhou et al., 2011b). Notably, in the Downing et al. study, no interstrain expression differences in genes coding for alcohol or aldehyde dehydrogenase, or the p450 enzyme Cyp2e1 that is involved in alcohol metabolism, were found.

Epigenetic

Epigenetic modifications entail changes in gene expression via mechanisms other than changes in DNA sequences (Kobor and Weinberg, 2011). The gene-silencing or enhancing activity can result from methylation, histone modification, and the effects of micro RNAs. The latter are single-stranded, non-protein-coding RNA molecules of about 17–25 nucleotides in length that control gene networks by translation repression. On one hand, the expression of certain micro RNAs is controlled by DNA methylation and chromatin modifications, and on the other, micro RNAs can affect the methylation machinery and the expression of proteins involved in histone modifications. Epigenetic changes may remain for the duration of a cell's life through many mitotic cell divisions, for multiple cell generations, and may also be passed on transgenerationally following meiotic cell division. It is notable that, as early as 1913, Dr. Charles Stockard concluded from studies of rats that alcohol had a paternally mediated transgenerational effect on perinatal mortality (Stockard, 1913).

In recent teratogenesis studies employing mice that have an epigentically sensitive allele as a biomarker, alcohol has been shown to cause hypermethylation and gene silencing, resulting in phenotypic changes (Kaminen-Ahola et al., 2010a, b). More specifically, mice carrying the Agouti viable yellow (A^{vy}) allele as a biomarker normally show variable coat colors, ranging from yellow to dark brown. In yellow mice the Agouti gene regulation region is hypomethylated and the gene is active, while in brown mice, the gene is silent as a result of a highly methylated regulation region. In mice that had been exposed to alcohol prenatally from the time of fertilization to GD 8 (comparable in humans to fertilization until early in the fourth week), increased methylation occurred, as indicated by darker coat colors.

Additionally, studies examining alcohol's effect on developing neurons and neuronal stem cells have shown epigenetic changes. More specifically, in cerebellar neurons from third-trimester equivalent alcohol-exposed rats, reductions were identified in histone acetylation and CREB-binding protein (CBP) (Guo et al., 2011). CBP is a histone acetyltransferase and a component of the epigenetic mechanism that controls neuronal gene expression. In cultured neural stem cells, a binge-like alcohol exposure paradigm prevented normal DNA methylation programming of key neural stem cell genes and retarded cell differentiation (Zhou et al., 2011a). In second-trimester human fetal brain neurospheres alcohol has also been shown to alter the fate of neuronal stem and progenitor cells (Vangipuram and Lyman, 2010).

Regarding micro RNAs, a relatively small subset of those expressed at various developmental stages show alcohol sensitivity (Sathyan et al., 2007; Miranda, 2012). Among the sensitive molecules are those in the miR-9 family, which plays a significant role in early neural tube patterning and telencephalic neurogenesis. During early prenatal development, miR-9 expression is diminished by alcohol, while at later developmental

stages and in adults it is induced. Alcohol is hypothesized to affect micro RNA expression by regulating the epigenetic landscape of the genome via alteration of methylation patterns and also by influencing the activity of ligand-gated ion channels.

Of interest, transgenerational alcohol-induced epigenetic modifications have been reported in a rat model. Offspring of rats fed an alcohol-containing liquid diet from GD 7 to GD 21 (corresponding to weeks 3–17 of human prenatal development) showed a deficit in proopiomelanocortin (Pomc)-related neural functions along with hypermethylationin of the *Pomc* promoter and altered levels of histone-modifying proteins and DNA methyltransferase levels in POMC neurons. These Pomc gene methylation and expression effects and functional deficits persisted in the male germline. Speculation has been advanced regarding a role for these prenatal alcohol exposure-induced changes in genetic and sex-related risks for alcoholism (Govorko et al., 2012).

Additional support for an epigenetic role in alcohol-induced neuroteratogenesis stems from rodent studies in which choline supplementation has been shown to be ameliorative. This effect is presumably mediated through the effects of choline on DNA methylation (Thomas et al., 2004, 2007, 2009, 2010; Ryan et al., 2008; Monk et al., 2012; Thomas and Tran, 2012). Indeed, a recent study entailing administration of this nutrient to alcohol-exposed neonatal rats and subsequent assessment of the brains of these animals on postnatal day 21 found that alcohol treatment-induced hypermethylation in the hippocampus and prefrontal cortex was reduced as a result of choline supplementation (Otero et al., 2012). Deficiency of another nutrient, folic acid, which is important in the production of methyl groups as are required for DNA methylation, has also been suggested as playing a role in alcohol's teratogenesis via an epigenetic mechanism (Ballard et al., 2012).

REACTIVE OXYGEN SPECIES-MEDIATED DAMAGE

Free radicals are highly reactive molecules, many of which contain oxygen. In cells they can cause oxidative stress, subsequent damage to cellular components, including membranes, DNA, and proteins, and can induce apoptosis.

There is considerable evidence supporting a role for oxidative stress in alcohol's teratogenesis, including a number of studies illustrating the protective capacity of both exogenous and endogenous antioxidants (Davis et al., 1990; Kotch et al., 1995; Chen and Sulik, 1996, 2000; Henderson et al., 1999; Ramachandran et al., 2003; Chen et al., 2004; Heaton et al., 2006; Wentzel and Eriksson, 2006; Parnell et al., 2010; Antonio et al., 2011). Regarding the latter, studies of cultured fetal rat cortical neurons suggest that alcohol causes glutathione depletion and that the highly differing sensitivity to alcohol-induced cell death within specific neuronal populations is related to their glutathione content (Maffi et al., 2008; Narasimhan et al., 2011). Importantly, chemically or virally induced transcriptional activation of Nrf2, a transcription factor that regulates the induction of detoxifying and antioxidant genes, has been shown to prevent alcohol-induced glutathione depletion and the related apoptotic death of cultured cortical neurons (Narasimhan et al., 2011); to protect against alcohol-induced oxidative stress and apoptosis in early mouse embryos (Dong et al., 2008, 2010; Yan et al., 2010); and to increase the survival of cerebellar granule neurons in third-trimester equivalent alcohol-exposed rats (Kumar et al., 2011). The effectiveness of antioxidants affords a practical, nutrition-based approach to reducing alcohol's teratogenicity in human populations.

Many pathways have been suggested as contributing to the ability of alcohol to induce a state of oxidative stress, with potential sources of radical oxygen species (ROS) including mitochondrial respiratory chain enzymes, xanthine oxidase, cytochrome P450 enzymes and NADPH oxidase (NOX). Recent studies of early mouse embryos have identified the latter as playing an important role in alcohol's teratogenicity, with alcohol exposure inducing NOX enzyme activity and increasing mRNA expression of NOX regulatory subunits. The NOX inhibitor, diphenyleneiodonium, was shown to be protective, diminishing alcohol-induced ROS generation, caspase-3 activity, and apoptosis (Dong et al., 2010).

RETINOID AND SONIC HEDGEHOG SIGNALING

Retinoic acid (RA) signaling is critical for normal development (Rhinn and Dolle, 2012), with both diminished and excessive RA concentrations resulting in birth defects. Recognizing that RA synthesis depends on oxidation of retinol and retinal by enzymes that also metabolize alcohol (alcohol and aldehyde dehydrogenase), Pullarkat (1991) and Duester (1991) hypothesized that alcohol's teratogenicity results from its competitive inhibition of these enzymes. Supporting this as a potential mechanism is work by Deltour et al. (1996) demonstrating in cultured mouse embryos that alcohol exposure reduces RA concentrations. Additionally, in several developmental systems RA receptors (RARs) and cellular retinol-binding protein are disregulated by alcohol (Zachman and Grummer, 1998). Illustrating RA

involvement in alcohol teratogenicity, Johnson et al. (2007) employed a pan-RAR antagonist and disulfiram, an ALDH inhibitor, showing that temporary abrogation of RA signaling results in limb defects comparable to those resulting from alcohol exposure. In these experiments RA was shown to diminish excessive cell death as underlies alcohol-induced limb defects; and alcohol was shown to reduce the expression of sonic hedgehog (Shh), a key developmental control gene. Previous work had shown that Shh signaling is stimulated by, and dependent upon, RA (Helms et al., 1994; Stratford et al., 1996).

Mounting evidence implicates interference with Shh signaling in alcohol-induced birth defects, especially those resulting from early gestational exposure. Studies conducted using an acute (high-binge) exposure mouse model have shown that the brain and face are concurrently affected following alcohol insult occurring at early gastrulation stages of development (corresponding to the third week postfertilization in humans) and that the widely ranging degrees of effect include all of the salient craniofacial features of FAS and corpus callosum abnormalities as also characterize the severe end of FASD (Sulik et al., 1988; Sulik, 2005; O'Leary-Moore et al., 2011) (Figs. 26.3 and 26.4). Severely affected animals present with median facial and forebrain deficiencies that fall within the spectrum termed holoprosencephaly. In humans holoprosencephaly is recognized as resulting from environmental exposures and/or genetic abnormalities involving the Shh gene itself, as well as genes that modulate the Shh pathway (Roessler and Muenke, 2010; Solomon et al., 2012). Studies employing fish and chick models have shown that provision of exogenous shh protein (Ahlgren et al., 2002) or Shh mRNA overexpression (Loucks and Ahlgren, 2009) in alcohol-exposed embryos prevents many of the gross physical phenotypes, suggesting that the suppression of Shh signaling, as is required for normal ventral forebrain induction and development, is one of the major effects of alcohol exposure. Supporting this premise is recent research showing that the severity and incidence of FAS-like facial and brain abnormalities, including those falling within the holoprosencephaly spectrum, are exacerbated in prenatal alcohol-exposed mice by haploinsufficiency of Shh as well as Gli2, which is a transcriptional activator in the Shh pathway (Kietzman et al, 2014). Additionally, mutation of the Shh coreceptor, Cdon, synergizes with alcohol to yield holoprosencephaly in mice (Fig. 26.5) (Hong and Krauss, 2012). While the molecular mechanism of alcohol's synergistic action remains unknown, findings from these gene–environment interaction studies highlight the promise of continuing to seek genes whose modification/mutation may exacerbate or attenuate alcohol's impact on development.

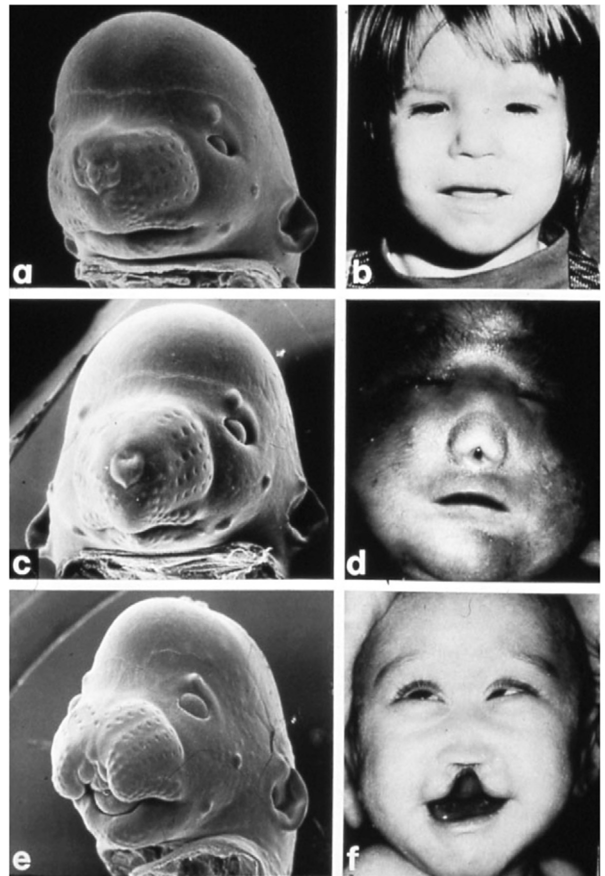

Fig. 26.3. The facial features of mouse fetuses acutely exposed to alcohol on their 7th day of gestation (comparable to the mid third week of human development), showing varying degrees of effect (**a**, **c**, and **e**), can be compared with those of an individual with fetal alcohol syndrome (**b**) and those whose defects are recognized as falling within the holoprosencephaly spectrum (**d** and **f**). (Modified from Sulik, 2005.)

CONCLUSION

Animal model-based studies have provided a wealth of information regarding the genesis of prenatal alcohol exposure-induced structural and functional abnormalities and are expected to continue to play a prominent role in this regard. As evidenced by the selected studies considered herein, a number of differing teratogenic cascades account for a wide variety of alcohol-induced developmental endpoints, with the developmental stage and genetic background of the exposed individual as well as alcohol exposure patterns and dosages playing critical modulating roles. Overall, it is clear that the developing brain is vulnerable to alcohol insult at virtually every prenatal stage. Technologic advances and rapidly increasing knowledge in the fields of genetics, cell, developmental, and neurobiology are essential to accurately piecing together experimental evidence in refining

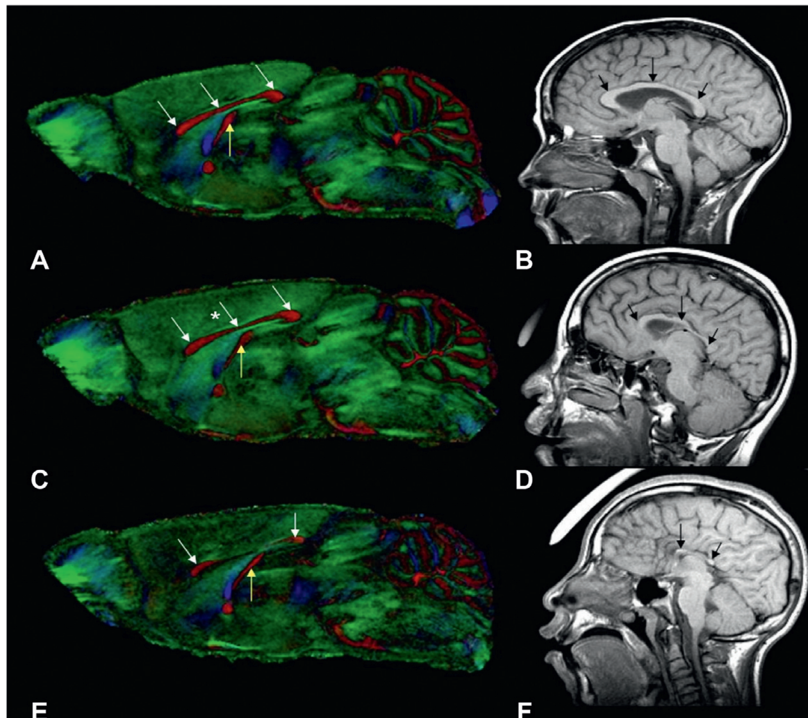

Fig. 26.4. Alcohol exposure on gestational day (GD) 7 (comparable to the mid third week of human development) in mice yields permanent central nervous system dysmorphology comparable to that in individuals with fetal alcohol syndrome (FAS). Diffusion tensor images are from a control adolescent mouse (**A**) and two that had been alcohol-exposed on GD 7 (**C**, **E**). The alcohol-exposed mice show varying degrees of brain dysmorphology, including thinning (star in **C**) to median absence of the corpus callosum (**E**). The hippocampal commissure (yellow arrows) is also reduced in the more severely affected mouse (**E**). Dysmorphology of the corpus callosum in this mouse model is remarkably similar to that seen in individuals with FAS. As compared to a control individual (**B**), those with FAS (**D**, **F**) also have considerable dysmorphology of the corpus callosum. (Human images courtesy of Dr. S. Mattson. Reproduced from O'Leary-Moore et al., 2011, by permission of Springer Science and Business Media.)

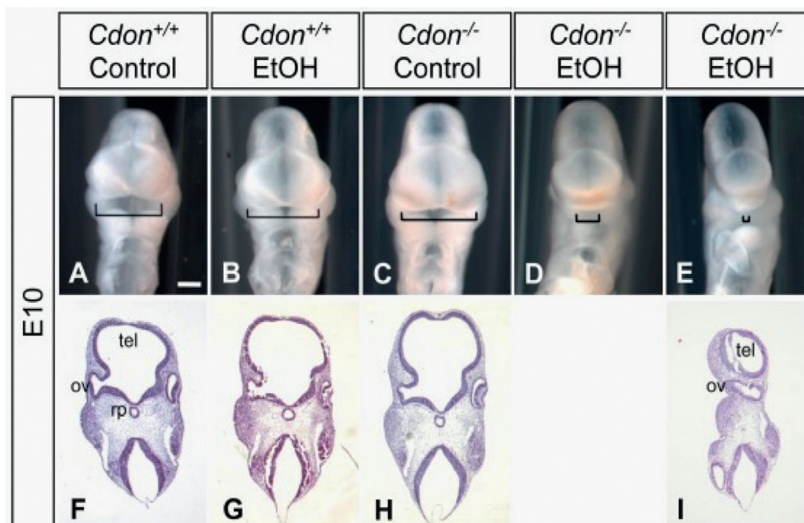

Fig. 26.5. Synergy between loss of Cdon, which encodes a Shh coreceptor, and prenatal alcohol exposure produces holoprosencephaly. (**A–E**) Frontal views of gestational day (GD) 10.0 mouse embryos (comparable to week 5 of human development) of the indicated genotype that had been treated *in utero* on GD 7 (comparable to the mid third week of human development) with alcohol (EtOH) or saline (control). Brackets indicate distance between the left and right nasal pits, a measure of midline formation. Scale bar, 250 mm. (**F–I**) Coronal sections stained with hematoxylin and eosin. Some alcohol-treated Cdon2/2 embryos (**E**, **I**) displayed severe holoprosencephaly with loss of telencephalic structure (tel), a single optic vesicle (ov), and absence of the anterior pituitary precursor, Rathke's pouch (rp). (Reproduced from Hong and Krauss, 2012.)

our understanding of the genesis of alcohol-induced birth defects, to the planning and execution of future studies, and to applying the knowledge gained to diminish the severity or occurrence of FASD.

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