Chapter 5
Mechanisms of Teratogenesis

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Historical Recognition of Deficits Associated with Prenatal Alcohol Exposure

The diagnosis of "Fetal Alcohol Syndrome" (FAS) was first clinically defined in a British medical journal, *The Lancet*, in 1973 [1]. However, observational links between ethanol exposure during pregnancy and adverse health outcomes have been documented for centuries. Published historical reports have cited the Old Testament[1] and lectures by ancient Greek and Roman philosophers,[2] including Aristotle, as the first written acknowledgments of alcohol's teratogenic effects on a developing fetus

[1] ***Judges 13:3–4***, The angel of the Lord appeared to her and said, "You are barren and childless, but you are going to become pregnant and give birth to a son. Now see to it that you drink no wine or other fermented drink and that you do not eat anything unclean."

[2] In *Anatomy of Melancholia* (1621), Robert Burton quotes Roman author Aulus Gellius (130–180 AD): "If a drunken man get a child, it will never likely have a good brain" and Greek philosophers Plutarch (~120 AD): "one drunkard begets another" and Aristotle (322 BC): "foolish, drunken or hare-brain women, most part bring forth children like unto themselves." Plutarch further describes lawgiver Lycurgus' advice for child-rearing in ancient Sparta in *Life of Lycurgus* (reproduced in 1914): "In order to the good education of their youth (which, as I said before, he thought the most important and noblest work of a lawgiver), he [Lycurgus] went so far back as to take into consideration their very conception and birth, by regulating their marriages… he had tried all ways to reduce the women to more modesty and sobriety…" This thinking may have influenced ancient laws in the cities of Carthage and Sparta, which prohibited the use of alcohol by newly married couples to prevent conception during intoxication, according to Warner & Rosett, *The Effects of Drinking on Offspring* (1975).

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[2, 3]. Artist William Hogarth painted "Gin Lane" in 1751 in response to England's Gin Epidemic, possibly one of the earliest artistic renderings of harm to children following prenatal alcohol exposure. The painting centers on a mother engaging in "exuberant drinking" while dropping a baby with notable facial malformations, which are now recognized as a symptom of FAS. Within the next century, recognition of alcohol as a teratogen would appear in academic works, including Charles Darwin's "On the Origin of Species," as well as in popular literature, including Charles Dickens' novel *The Posthumous Papers of the Pickwick Club*.[3] Indeed, by the time Aldous Huxley references a child who is "stunted" by prenatal alcohol exposure in his 1932 novel *Brave New World*, there appears to be a societal consensus that alcohol exposure during pregnancy can produce negative developmental outcomes.

At the turn of the twentieth century, clinical researchers associated intrauterine alcohol exposure with distinct health risks during pregnancy and birthing, including spontaneous abortion, premature labor, and higher mortality rates in newborns [3]. Furthermore, the ability of alcohol to cross the placental barrier, and to be transmitted through breast milk, was discovered in 1900. A boom of research incorporating animal models, leading up through 1922, reported that offspring exhibited physical malformations after birth and throughout the lifespan. However, with the onset of Prohibition in the 1920s, research into the harmful effects of alcohol exposure declined suddenly and considerably, viewed unnecessary following the ban of alcohol consumption in the United States. This mindset carried through to the 1950s, well after the repeal of the 21st Amendment, when alcohol consumption among the general population was once again socially accepted. It was recognition of physical malformations in developing children exposed to radiation—specifically, explosions in Hiroshima and Nagasaki [4]—as well as widespread prescription of *thalidomide* to pregnant women in the 1950s–60s [5], that caused the resurgence of research into teratogenic exposures during pregnancy. Still, research investigating prenatal alcohol exposure remained minimal until Jones and Smith defined FAS in 1973, supplementing their diagnostic criteria with supporting evidence of prenatal alcohol exposure-induced deficits in humans. Follow-up investigations by Jones' research group incorporated animal models and accounted for confounding environmental and socioeconomic variables, providing compelling evidence that prenatal alcohol exposure directly produced not only physical impairments in exposed offspring, but behavioral deficits as well.

[3] Dickens writes, "Betsy Martin, widow, one child, and one eye. Goes out charring and washing, by the day; never had more than one eye, but knows her mother drank bottled stout, and shouldn't wonder if that caused it… Thinks it not impossible that if she had always abstained from spirits she might have had two eyes by this time."

Investigating the Toxic and Teratogenic Effects of Prenatal Alcohol Exposure: Factors of Consideration in Research

Research Models and Subjects

Just as our societal views on alcohol consumption during pregnancy have changed, so has the strict classification of alcohol as a poisonous agent. It is tempting to assume, based on observable deficits in children with a history of prenatal alcohol exposure, that alcohol behaves exclusively as a toxin. However, the field of fetal alcohol spectrum disorder (FASD) research has advanced to recognize that not all of alcohol's effects are toxic—rather, prenatal alcohol can also contribute to the adaptive reprogramming of cells and tissues, without directly inhibiting cell survival (a.k.a., *teratology*), which we will describe in this chapter.

It is important to acknowledge that the history of research investigating prenatal alcohol exposure began with clinical observation—recognizing an association between fetal alcohol exposure and distinct morphological and behavioral phenotypes in these same children after birth [1, 6]. These observations have led to numerous associative studies in humans—from reduced arousal and reflex scores on the Brazelton Newborn Behavioral Assessment Scale, to abnormal development of frontal lobe gray and white matter in magnetic resonance and diffusion tension imaging [7]—which have informed our ability to recognize and diagnose FASD in clinical settings. However, there are also a vast number of studies that incorporate non-human subjects, including simple systems (cells, organoids, and tissues) and living animals, to investigate prenatal alcohol teratology. These investigations have been crucial for identifying and manipulating biological mechanisms impaired by intrauterine alcohol exposure. There are several benefits and practical reasons for performing these types of non-human research.

First, no single medical assessment can definitively diagnose an individual with FASD; often, diagnoses rely on self-reports of prenatal alcohol exposure by pregnant individuals. Unfortunately, such behaviors are largely unreported, in part due to social stigmatization and shame. In a 2017 survey of adults, mothers of children with FASD were perceived with more disdain than women with mental illnesses, substance use disorders, and histories of incarceration [8]. In experiments incorporating animal subjects, exposures to alcohol are tightly regulated, allowing researchers to obfuscate the ethical and social concerns with prenatal alcohol exposure in humans. Another notable consideration of data collected from human subjects is that relationships between prenatal alcohol exposure and reported symptoms are associative, but not necessarily causal. In real-world cases of FASD, child outcomes often reflect comorbidities (i.e., high maternal stress, neglect, poly-drug exposures) which confound our ability to distinguish which observed outcomes are attributable to a history of alcohol exposure. Preclinical research allows for highly controlled exposure paradigms, which may examine these co-exposures and environmental factors in combination with prenatal alcohol exposure while incorporating appropriate control groups/conditions. Finally, from the breadth of existing research, we

have observed common features of prenatal alcohol exposure across animal models—including non-human primates, rodents, sheep, and zebrafish—which correspond with features observed in humans (see review [9]). With the added benefit of shorter gestational periods, as well as the potential for using invasive techniques to identify mechanistic contributions to offspring outcomes (i.e., physiology, histology, and genetics), animals provide unique benefits and insights for researchers, informing future therapeutic and clinical treatments of FASD symptoms.

In addition to animal models, cells derived from humans and animals can serve as the simplest model for investigating alcohol's effects on cellular processes, including differentiation, proliferation, and programmed cell death (apoptosis). In these models, alcohol can be applied acutely to cell cultures derived from the neuroepithelium, whole brain extracts, or neuronal/glial cell lines. Human-induced pluripotent stem cells (iPSCs), for instance, can be differentiated into every cell type of the human body, and allow for in vitro investigations of alcohol's toxicity to different subcellular populations. Prior research of iPSC-derived neurospheres—three-dimensional systems of clustered neural precursor cells—has demonstrated that alcohol exposure causes premature apoptosis [10]. Furthermore, human iPSCs can be grown to generate cerebral organoids ("mini brains"), which allow cells to differentiate into layers which structurally mimic real, developing brains. Although these cell lines are the least translational models to humans, in terms of the complexity of the FASD phenotype, cell culture investigations have been pivotal to scientists' understanding of alcohol-induced changes to epigenetic profiles, cell cycle function, and transcriptional regulation during development [11].

Dose/Levels of Alcohol Exposure During Pregnancy

One challenge faced by researchers investigating prenatal alcohol exposure is factoring in the amount of alcohol consumed at a particular time point during pregnancy. People often underestimate their levels of consumption [12], in part due to the variety of cup/glass sizes (i.e., pint, bottle, wine glass), variation in how much beverage is poured into a glass, and a lack of knowledge about the alcohol by volume (ABV) associated with a particular beverage (for instance, beer has a substantially lower ABV than wine, which in turn has a much lower ABV than spirits). To properly assess one's alcohol consumption, an individual would have to multiply their beverage's ABV by the total volume of liquid, and sum all beverages into alcohol "units." Not only is this uncommon by everyday drinkers, but when we consider the sedative effects of alcohol that lead to memory impairments, which are also dose dependent [13], its unsurprising that drinkers often struggle to accurately self-report how much alcohol they have consumed.

Despite the difficulties with reliably assessing alcohol consumption, existing literature has demonstrated that the amount of alcohol fetuses are exposed to dictates the extent of their impairments, across a multitude of measures. Newborns exposed to >1 drink per day during the first trimester of pregnancy were more likely to

express FASD symptoms, including lower birth weight, shorter body length, and increased facial dysmorphology, compared to newborns exposed to <1 drink per day [14]; importantly, infants exposed to these lower alcohol levels still exhibited FASD symptoms compared to non-exposed infants. To more accurately determine the effects of alcohol dose on offspring survival, Clarren and colleagues [15] performed a controlled series of experiments in pregnant macaques investigating the effects of a range of alcohol doses, delivered once a week throughout gestation. Alcohol exposures that produced blood alcohol levels of at least 205 mg/dL significantly increased the risk for spontaneous abortion. (For reference, 205 mg/dL can be achieved by a 150 lb woman after 7 drinks, with drinks defined as 1.25 oz of liquor, 12 oz of beer, or 5 oz of wine [16].) Furthermore, as blood alcohol levels continued to increase, pregnant individuals experienced even greater risk of pregnancy loss, with 83% of pregnancies ending early when alcohol levels exceeded 250 mg/dL. In the United States, a blood alcohol level above 80 mg/dL is legally qualified as intoxication; however, humans are capable of drinking considerably more (exceeding levels of 400 mg/dL [17]), depending upon the individual and the amount of alcohol consumed.

Importantly, 80 mg/dL is not a cutoff for "safe" alcohol exposure during pregnancy. In a series of experiments performed in rodents, pregnant females received liquid diets of 2%, 3%, and 5% ethanol throughout gestation, leading to average blood alcohol levels of 7, 30, and 83 mg/dL, respectively. Importantly, when their exposed offspring grew into adulthood, they demonstrated dose-dependent deficits in hippocampal synaptic activity and spatial learning tasks, with deficits increasing incrementally with dose [18]. These data are further supported by research in humans, with pregnant individuals who report low, moderate, or high levels of alcohol use: meta-analyses have demonstrated that even low-moderate exposures impact child development, cognitive performance, and mental health [19, 20]. Although considerably more research is necessary to investigate the consequences of sub-intoxicating prenatal exposures, many scientists today challenge the notion of a "safe" level of alcohol exposure during pregnancy.

Timing and Frequency of Alcohol Exposure During Pregnancy

Another factor to consider in understanding alcohol's impact on a developing fetus is the time during gestation in which alcohol exposure occurs. During the first few weeks after fertilization, alcohol exposure can impede the proper implantation of a developing blastocyst in a woman's uterus, resulting in early termination of a pregnancy even prior to detection [21]. Within 4–6 weeks after fertilization, differentiation of cardiac myocytes occurs, and alcohol exposure can inhibit proper growth, migration, and specification of cardiac progenitor cells. Furthermore, during this period of time, alcohol exposure can damage neural progenitor cells, which has been tied to the facial dysmorphology associated with FAS [21]. This window of development for facial features may carry into the second half of the first trimester,

as alcohol exposure within this time period corresponds with incidence of a smooth philtrum and thin vermillion border in exposed children [14]. Fetal exposure to alcohol is most common during the first month of pregnancy [22], likely because the pregnancy has not been detected, and alcohol exposure during this month of life has been associated with low infant birth weight, reduced body length, and smaller head circumference [14]. Importantly, there are numerous critical periods of development for embryonic tissues and structures within each gestational trimester. Preclinical and clinical research have cohesively informed the theory that fetal organs and tissues forming at the time of alcohol exposure are particularly vulnerable to long-term damage [23–25].

Related to the timing of alcohol exposure, the frequency of alcohol exposure during pregnancy also corresponds with the degree of impairment observed in exposed children. In a series of experiments by Clarren and colleagues, pregnant female macaques were exposed to alcohol once a week during the first 3 or 6 weeks of pregnancy or for the entire 24 weeks of gestation [26]. Offspring exposed to either 6 or 24 weeks of alcohol exposure experienced significant delays in memory and learning tasks, which were most pronounced in the offspring exposed for 24-weeks, as well as greater difficulty walking and climbing compared to both 3-week-alcohol-exposed and non-exposed offspring [26]. Importantly, these experiments demonstrated that early-gestation alcohol exposure (6 weeks) followed by abstinence failed to recover deficits in offspring, producing cognitive and behavioral outcomes comparable to exposure throughout pregnancy. However, these data should be interpreted with caution, as research in humans has found that in several measures of FAS—including infant growth and neurodevelopmental outcomes—avoiding alcohol exposure early in pregnancy can reduce the severity of symptoms expressed by children [27]. This conclusion was supported regardless of whether developing fetuses were exposed to low-moderate or high alcohol levels during pregnancy. Indeed, when alcohol exposure was significantly reduced or eliminated within the first ~6 weeks of pregnancy, children exhibited less severe FAS symptoms than those who were exposed to alcohol throughout pregnancy.

Taken together, there does not appear to be a point during embryonic development in which prenatal alcohol exposure lacks consequences. Rather, specific deficits expressed by children may correspond with the timing of exposure, as well as the amount and frequency of alcohol exposure during pregnancy.

The Toxicology of Prenatal Alcohol Exposure

Across diverse exposure paradigms and subject models, a substantial body of literature has demonstrated that alcohol can be toxic to an exposed organism, contributing to the physical, mental, and behavioral abnormalities that we associate with FASD [28]. Here, we will briefly discuss mechanisms commonly associated with the toxicity of ethanol, including increased oxidative stress, mitochondrial damage, and apoptosis/cell death (Fig. 5.1).

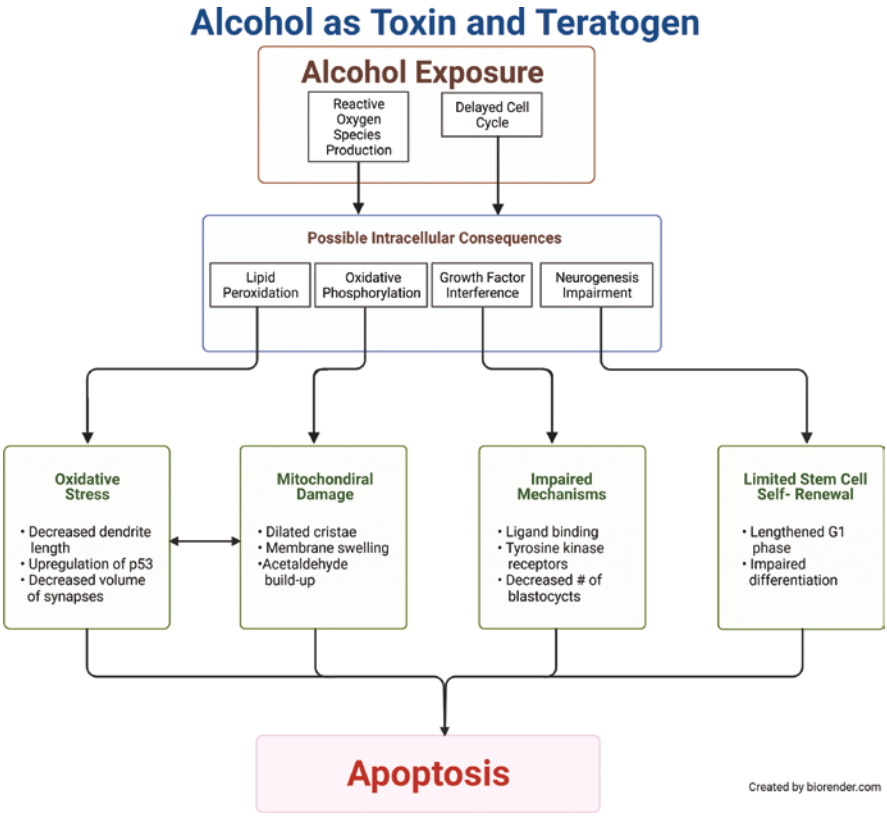

Fig. 5.1 Alcohol as a toxin and teratogen. This figure summarizes alcohol's direct effects as a poisonous toxin, as well as indirect effects as a teratogen, which contribute to increased rates of apoptosis following prenatal alcohol exposure

Oxidative Stress

Oxygen is required for molecular electron transfer and energy (or ATP) production in aerobic species. However, excess amounts of oxygen can produce harmful side effects, such as oxidative stress. Oxidative stress is the imbalance of reactive oxygen species (ROS) and antioxidant defenses [29] and serves as a driving mechanism to change mitochondrial structure/function, and damage lipids, proteins, and DNA [30–34]. Such imbalance caused by oxidative stress can lead to free radicals damaging DNA, proteins, and fatty tissue in an individual, with prolonged damage leading to a variety of diseases over time [35]. Prenatal alcohol exposure has a direct relationship with the aggregation of free radicals, with alcohol causing an increase in free radicals, ROS, and, consequently, oxidative stress [36, 37]. When free radicals attack other biological molecules—for example, lipids—they can damage cell membranes and induce lipid peroxidation [30, 33, 35]. Lipid peroxidation is often used as an indicator of oxidative stress, as this chain reaction of lipid degeneration

results in high volumes of ROS [38]. The increase in ROS and subsequent oxidative stress can potentially, along with other factors, cause neuronal deficits that are associated with FASD [39].

Nicotinamide adenine dinucleotide phosphate (NADPH) is another ROS that can initiate reactions to increase lipid peroxidation by generating high quantities of oxidoreductases during early pregnancy, including the enzyme family of cytochrome P450, which are directly involved in the metabolism of alcohol [35, 40]. Greater lipid peroxidation can be detrimental to structures required for *in utero* synapse formation during early development as well [41]. An *in vitro* rat hippocampus study looking at dendrites and their synapses showed that, without affecting cell survival, six days of alcohol exposure resulted in an overall decrease in the total number, length, and synapse of dendrites [42]. Taken together, these further support the negative effects, through higher levels of oxidative stress and lipid peroxidation, that alcohol can have on a fetus during the early gestational periods [43].

Aldehydes formed through lipid peroxidation reactions, as well as through the metabolism of alcohol, also contribute to increased productions of ROS and can damage proteins in the process [31, 36]. While research attempting to identify specific proteins damaged by excess ROS is ongoing, such damage compromises the proteins' overall structures, which can prevent normal cellular metabolism [31]. During the metabolism of alcohol, acetaldehyde is oxidized to acetate along one of the cell's metabolic pathways, which increases respiratory chain activity and can once again lead to greater oxidative stress through the overproduction of ROS [36]. Alcohol exposure also directly contributes to reductions in the number of neurons throughout multiple brain regions, by causing an immediate increase in ROS and oxidative stress, leading to mitochondria-mediated apoptotic cell death of neurons [44, 45].

In conclusion, alcohol-induced oxidative stress negatively alters the role that oxygen plays within the cell, leading to the hindrance of other intracellular mechanisms that contribute to the expression of FASD symptoms [46]. Further research is necessary to explore these respiratory reactions, and specifically how they can be stimulated to self-repair following alcohol exposure. This research would inform therapeutic interventions to potentially minimize the damage of oxidative stress during fetal development.

Mitochondrial Damage

Mitochondria are organelles essential for the production of ATP, for maintaining reduction potential and ionic balance, and for normally-occurring apoptosis. They are also crucial in supporting multiple cellular signaling pathways, cell-cell communication, and overall healthy cellular function [47]. When mitochondria are damaged, such as following alcohol exposure, the cell's primary energy source is compromised, increasing the probability of ROS developments (and possibly the

overproduction of ROS), disturbing ionic balances, and inducing inappropriate apoptosis [47]. Alcohol exposure has toxic effects on both the structure and function of the mitochondria [48, 49], producing elongation, cristae (the folds of the inner mitochondrial membrane), disorientation, and overcrowding of material within the mitochondrial matrix [50]. Cumulatively, this means that alcohol exposure can disrupt mitochondrial functions of generating ATP and cell-cell communication [51, 52].

Additionally, alcohol exposure inhibits the function of the electron transport chain (ETC) located in the inner mitochondrial membrane [51, 52], which can lead to the overproduction of ROS [53, 54]. Subsequently, stray electrons escape the ETC and react with oxygen outside the ETC to form superoxide or hydrogen peroxide; this is why the mitochondria is the primary source of ROS [53, 54]. This increase in ROS removes electrons ("oxidizes") from the ETC complex subunits, which results in oxidative phosphorylation and decreased ATP levels, leading to insufficient mitochondrial energy production [51, 52]. In response to the impaired production of ATP, the internal integrity of the mitochondria is compromised, and the cristae become dilated [55, 56]. Because the proper formation and folding of cristae are crucial for its function and capacity to synthesize ATP, dilated cristae are associated with oxidative stress through either elevated ROS production or reductions in ROS protective mechanisms, i.e., antioxidants [43, 57]. Importantly, this connection between dilated cristae and ROS overproduction caused by alcohol exposure illustrates how alcohol can directly induce oxidative stress and lead to mitochondrial damage. Furthermore, there is evidence that the introduction of foreign substances (such as alcohol) leads to a mitochondrial response which incites an independent increase in ROS production and oxidative stress [55, 58]. Together, these findings imply that ethanol exacerbates the relationship between oxidative stress and mitochondrial damage.

Furthermore, alcohol exposure reduces mitochondrial glutathione, an antioxidant, and may lead to more oxidative damage, including lipid peroxidation, that changes how much ATP the mitochondria synthesizes [51, 57]. There is additional evidence that alcohol alters various signaling pathways to the mitochondria, including Complex I, Complex IV, succinate dehydrogenase, and ADP translocase activities. These changes produce a rapid onset of oxidative stress that precedes cellular apoptosis [44, 48]. Lipid peroxidation also affects the permeability of mitochondrial membranes, which produces mitochondrial swelling. This swelling is a result of increased cytochrome c release, caspase activation, and DNA fragmentation that also can lead to increased rates of cellular death [51, 59, 60].

In summary, the mitochondria play an integral role in balancing out ROS production and limiting oxidative stress. When function of this organelle is impeded by alcohol, the cell becomes more susceptible to destructive reactions [61]. Research investigating therapeutic treatments that are specific to maintaining the functional and structural integrity of the mitochondria would yield valuable information for combatting the toxicity of alcohol exposure and ensuring the maintenance of healthy levels of ROS [51].

Apoptosis

Optimal brain development requires a homeostatic degree of programmed cell death (PCD), with apoptosis serving as the controlled process of cell death via specific cellular pathways. During apoptosis, various membrane receptors, such as Fas, TNFR, and cytochrome c, trigger signal transduction steps that activate cysteine proteases, i.e., caspases [30, 62–64]. The initiation of apoptosis involves the loss of cell division controls, interference with growth factors, changes to cell attachment to tissue surface, and the activation of specific proteins that trigger cell death [30, 65–67]. Cells undergoing apoptosis show membrane blebbing, chromatin condensation, and DNA fragmentation until the cells eventually break down into smaller membrane-bound fragments, apoptotic bodies, and are no longer viable [68–70]. PCD is essential in fetal development and organ formation, occurring throughout embryonic development in normally-developing children. However, excessive embryonic cell death can disrupt the formation of organs or tissues and cause structural or functional abnormalities [68, 71].

Alcohol, as a toxic substance, can affect specific tissues and cell types more than others, depending on a multitude of factors including the amount and timing of exposure during gestation [30, 72]. These critical developmental windows are an essential consideration when investigating the tissues or cell types that are most susceptible to harm following alcohol exposure. Importantly, because cells are already biologically primed for apoptosis, exposure to alcohol may cause a dramatic increase in cell death, especially during embryonic development [73]. Cellular impairment in response to alcohol exposure has been attributed to several deficiencies, including vitamin A compound levels, antioxidant compounds levels (high oxidative stress), and an interference with normal internal cellular communication pathways [30]. Retinoic acid (RA) is an active form of vitamin A and a key regulator of morphogenesis. Importantly, RA deficiencies are linked to increased apoptosis in neural crest cell populations [30, 74]. These deficiencies can be compounding factors to the negative effects of alcohol, including increased apoptosis, during development.

As stated previously, alcohol increases the abundance of free radicals that are associated with elevated levels of oxidative stress. These free radicals can further damage embryonic cells by inciting unnecessary apoptosis [30, 33]. In addition, cell membrane-associated proteins act as communication signals from the outside of the cell to the inside. Alcohol interferes with this process by inhibiting this central communication pathway of the cell (inhibiting intracellular signaling kinases or increasing intracellular calcium), augmenting further cell death [30, 75, 76]. There may also be a direct neurotoxic effect of alcohol on the nervous system, both inciting apoptosis and reducing the density of synapses [30]. Several studies have found higher rates of apoptosis and neural crest populations resulting from alcohol exposure [30, 33, 77], once again highlighting the vulnerability of specific brain regions and tissues in the central nervous system at different timepoints and gestational periods. It is important to note that this is not an exhaustive explanation of

alcohol-induced apoptosis, as research is currently underway to further understand the mechanisms through which alcohol increases rates of cell death. The goal of this research is to identify cellular targets with the potential to be therapeutically regulated, and to ultimately reduce the frequency and magnitude of physical, behavioral, and mental abnormalities in developing fetuses.

The Teratology of Prenatal Alcohol Exposure

Aside from its toxic effects, alcohol is also a prominent teratogen—a substance that disrupts normal fetal developmental pathways and programs. Prenatal alcohol exposure is the most prevalent cause of neurobehavioral deficits in Western countries [30], creating long-term cellular damage that contributes to the development of FASD. These lasting effects of alcohol exposure *in utero* can occur during the cell cycle or during stem cell self-renewal/growth (Fig. 5.2) and can interfere with

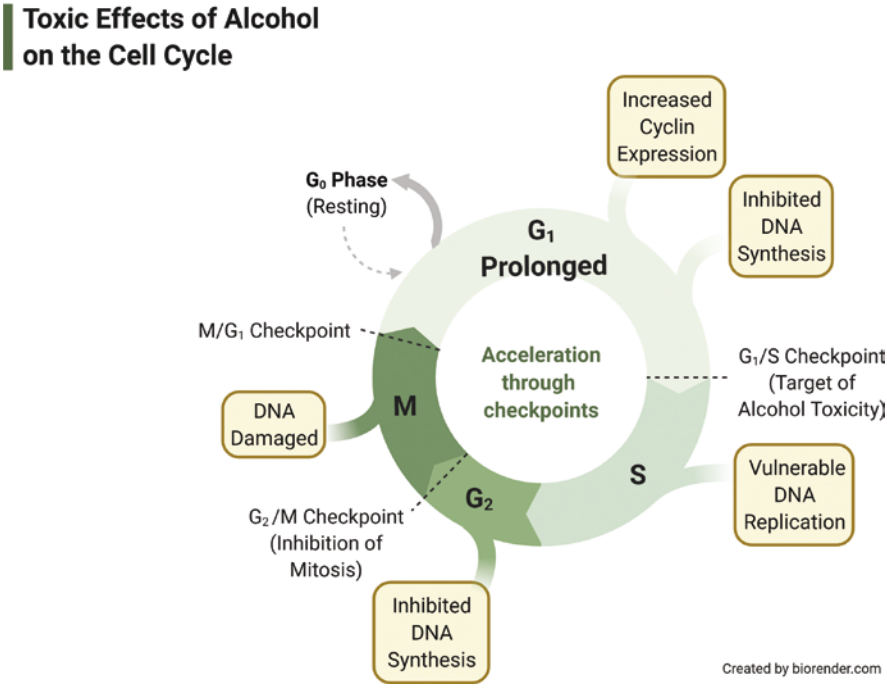

Fig. 5.2 The toxic effects of alcohol on the cell cycle. Alcohol toxicity impacts cellular function within each stage of the cell cycle, altering cell cycle progression and subsequent proliferation in exposed cells. These effects may furthermore be cell-type specific and dependent on the stage of development at which alcohol exposure occurs. G_0 phase: a class of cells that have the potential to divide but have not yet entered the cell cycle; interphase stages of the cell cycle—G_1: cell growth, S: DNA synthesis, G_2: preparation for mitosis, and M: mitotic

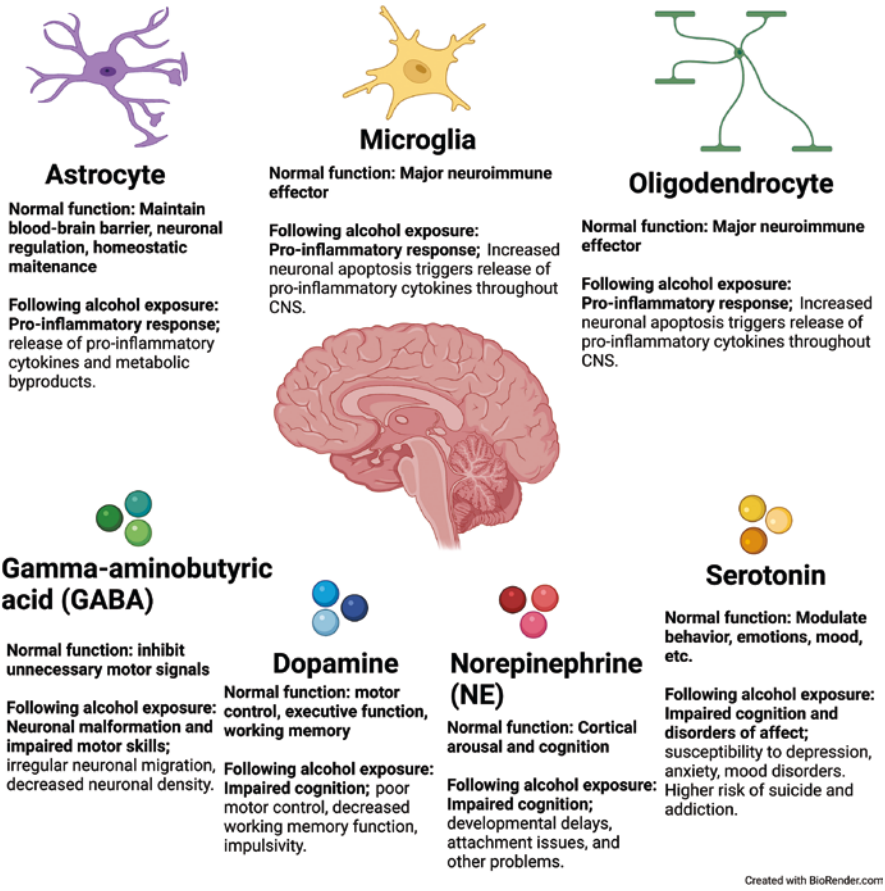

Fig. 5.3 A summary figure of the effects of prenatal alcohol exposure on glia and neurotransmitters

growth factor activities and neuronal activity, both directly and indirectly through regulation by glial cells (summarized in Fig. 5.3).

The Cell Cycle

The cell cycle is the multifaceted process in which cells grow (interphase) and divide (mitosis). The interphase stages of the cell cycle include G_1 (cell growth), S (DNA synthesis), G_2 (preparation for mitosis), and M (mitotic) phases (Fig. 5.2). The M phase, consisting of four sub-phases, prophase, metaphase, anaphase, and telophase, results in the division of a parent cell into two daughter cells. An additional G_0 phase encompasses a class of cells that have the potential to divide but

have not yet entered the cell cycle [78]. Cell cycle is very important in early embryonic development because the growth and duplication of DNA are crucial in transmitting generational information [79, 80]. It is vital for each cell to follow the proper order of the cell cycle to ensure each daughter cell has equally distributed DNA [79, 81], and there are certain embedded biological controls to verify that this process is undisturbed. However, when these controls are compromised, such as following alcohol exposure, cells will stall in certain cyclic phases depending on the duration, volume, and timing of alcohol exposure, resulting in apoptosis, premature maturation, or inappropriate differentiation [79].

Chronic alcohol exposure can have a lasting effect on cell cycle proteins and on cell proliferation. For instance, alcohol exposure induces an increase in G_1 cyclins and E2F Transcription Factor 1, which can alter cell cycle progression [82]. However, it has also been found that, depending on the cell type (such as neural progenitor cells), alcohol does not induce apoptosis but instead stimulates progression through the cell cycle [83]. An *in vitro* study of fetal cerebral cortical neuroepithelial cells found that alcohol exposure of 120 and 620 mg/dL, compared to alcohol-free controls, resulted in a significantly higher percentage of these cells in S-phase and G_2/M phase, without causing apoptosis [83]. The same study observed that these alcohol-exposed cells exhibited decreased proliferation capacity and asymmetric cell division of the stem cells, possibly depleting the fetal stem cell population. Contrastingly, in a study using neuronal-like cells, alcohol increased the amount of cells in the G_1 phase, but the proliferation capacity varied depending on the volume and length of alcohol exposure [72]. This implies that alcohol exposure prolongs time within the G_1 phase, possibly caused by a reduction in proliferation, the increase of apoptosis, and DNA damage, making the G_1/S checkpoint a prime target for alcohol toxicity [82, 84]. Additionally, there is evidence of slowed S-phase progression that is dose-dependent following exposure to alcohol [82], as well as inhibition of DNA synthesis and subsequently, mitosis [85]. Vulnerable tissue cells may also exhibit a prolonged G_2 phase, due to the diminished ability to repair DNA damage, and may eventually die from stalling at the G_2/M and G_0/G_1 checkpoints once exposed to alcohol [85]. Another study reports that alcohol exposure prolonged the G_2/M checkpoint due to the inactivation of the cyclin-dependent kinase protein, which functionally allows cells to progress through mitosis [86]. In summary, such diverse and seemingly contrasting alcohol effects on the cell cycle exemplify how various factors, including cell types and developmental periods, play a role in the immediate toxic effects of prenatal alcohol exposure on development, as well as long-lasting teratogenic effects on an individual with FASD.

DNA methylation is also compromised from exposure to alcohol [84]. The methylation of DNA is crucial to early fetal development because it facilitates embryonic cell differentiation while simultaneously protecting cells from regressing into an altered state [87, 88]. When cells experience a delay in any phase of the cell cycle, the potential for DNA damage and genetic mutations increases. However, the specific mechanisms responsible for delays in G_1 and S phases are still unknown and require further study.

Mechanisms for Stem Cell Self-Renewal and Growth

Stem cells may divide into daughter cells that either retain or loose the parent cell's potential to self-renew. Self-renewal is necessary to ensure that sufficient quantities of stem cells are retained throughout development and into adulthood [89]. Specific cellular mechanisms are responsible for promoting or limiting self-renewal, and maintaining the genomic integrity of stem cells. Disrupting these mechanisms may lead to inappropriate aging of stem cells, limiting their ability to grow, self-renew, and repair injury [89, 90]. Embryonic stem cells have the capacity to divide nearly endlessly, while preserving their self-renewal and differentiation potential [79]. Embryonic stem cells, like other self-renewing stem cells, possess a unique ability to maintain a state of proliferation [79], with rapid self-renewal of embryonic stem cells being associated with a shortened G_1 phase [91]. Because a shortened G_1 phase alters how fast and effectively embryonic stem cells grow, alcohol introduction may limit the stem cells' ability to fully and efficiently self-renew [91]. There are multiple immediate detrimental effects to embryonic stem cells, along with long-lasting effects that persist into adulthood in individuals who are exposed to alcohol during early development.

Immediate effects of prenatal alcohol exposure on fetal neural stem cells include the inhibition of microRNAs (miRNAs), DNA methylation, overproduction of ROS, and decreased neurogenesis. Exposure to alcohol may also have the potential to alter the migration, neuronal formation, and growth processes of neural stem cells' (NSCs) ability to self-renew or differentiate. It also prevents the cell from being able to repair itself when alcohol inhibits its primary function of self-renewing. Prenatal alcohol exposure has been found to hinder the expression of specific miRNAs (such as miR-9, miR-21, miR-153, and miR-335) that may help stem cells maintain their ability to self-renew [92–94]. These miRNAs are especially sensitive to alcohol and can cause premature differentiation in neural stem cells and negatively affect NSC populations [92]. Increased DNA methylation (or hypermethylation) also occurs when exposed to alcohol, which may further contribute to the reduction of NSCs that provide proteins to stimulate cell cycle phases (G_1 and G_2 especially) [95]. Oxidative stress and the overproduction of ROS also disrupt certain mechanisms for stem cell self-renewal and growth. This imbalance of ROS may suggest a decline in the function of stem cells that play a role in self-renewal, which is therefore altered due to alcohol exposure [96, 97]. Additionally, low levels of nicotinamide adenine dinucleotide (NAD+) may indicate the reduction of stem cell self-renewal and the differentiation of NSCs [96, 97]. Alcohol can also be detrimental to embryonic neurogenesis (the development of new neurons in specific brain regions) during early development. The regulation of neurogenesis is associated with intrinsic properties of NSCs, including cell surface receptors and intracellular signaling, that are negatively affected by alcohol [98]. Exposure to alcohol decreases NSC populations and causes them to become vulnerable as they mature. This can contribute to persistent neural abnormalities, leading to symptoms such as memory

deficits, which have been observed in cells with a history of intrauterine exposure to alcohol [99–101].

Alcohol effects on embryonic neurogenesis can potentially persevere and affect other developmental stages up to adulthood [98, 102]. An *in vitro* study using adult mice exposed to alcohol prenatally reported a decrease in neurospheres, neuronal differentiation, and overall neurogenesis in the adult hippocampus [98]. The decrease in hippocampal neurogenesis was not mediated by enriched environments, and was associated with impaired memory and learning functionality [102]. Furthermore, early postnatal alcohol exposure can result in lasting deficits of adult hippocampal neurogenesis that correspond with neuronal and behavioral deficits associated with FASD [103]. In addition, individuals with FASD may be predisposed to early onset cancer, higher chance of congenital heart disease, and impaired immunity as an adult, which may all contribute to a higher mortality rate due to the teratogenic effects of alcohol [93, 104–107]. Stem cells play a key role in preventing early aging by proliferating throughout each developmental stage. Alcohol disrupts this proliferative ability, acutely and persistently affecting stem cell function and in turn fetal development, eventually contributing to the behavioral and physical abnormalities linked to FASD.

Interference with the Activity of Growth Factors

Growth factors are secreted proteins that are released to influence the behavior of recipient cells, to facilitate cell division and differentiation, stimulate the development of tissues and organs, and protect against apoptosis and other mechanisms of cell death [108]. Prenatal alcohol exposure can disrupt growth factor expression and signaling [109–112] and consequently may prevent crucial cellular functions from occurring, including differentiation and cell division, or protecting against apoptosis [113–115].

The uterus is a source of a number of growth factors that have important mitogenic and differentiation effects on embryo and fetal maturation [114]. During embryonic development, blastocysts (small orbs of rapidly developing cells) divide into an inner group (or morula) and outer group (trophoblast) [116]. The inner group of cells eventually transform into embryonic cells [117]. Epidermal growth factor (EGF) increases blastocyst cell numbers, which reflects mitogenic and differentiating effects on fetal development [114]. Normal blastocyst and embryonic cell development are boosted by the production of growth factors throughout pregnancy, and when these growth factors are inhibited, it may alter the amount of blastocysts that contribute to fetal development [114]. Apoptosis in embryos negatively affects embryonic neurogenesis and consequently leads to an increase in oxidative stress in the placenta [109]. Alcohol exposure increases the amount of EGF-like growth factors, especially during increased levels of oxidative stress [109], perhaps as a compensatory mechanism to protect against apoptosis and oxidative stress [109]. Altered

expression of growth factors negatively influences fetal growth and development, which can eventually lead to FASD [118]. Brain-derived neurotrophic factor (BDNF), for example, a neurotrophic factor which plays a key role in maintaining the survival of neurons by promoting cell growth, differentiation, and maintenance to prevent apoptosis, was reported to be increased in amniotic fluid of small-for-gestational age fetuses [113], a common outcome following prenatal alcohol exposure. Such a response may constitute a maladaptation to an unhealthy environment and reflect an aberrant speeding up of cell maturation.

Lastly, Insulin-like growth factor (IGF)-I and II are associated with mediating neuronal growth and survival, metabolizing energy, and facilitating synapse formation [41]. Prenatal alcohol exposure has been shown to interfere with the activity of these growth factors as well. Specifically, alcohol interferes with growth factor signaling through impaired ligand binding and activation of receptor tyrosine kinases [41]. Ligand binding and tyrosine kinase receptors play a crucial role in the controlled growth and death of cells [119]. The impairment of these mechanisms causes the cells to become more susceptible to intracellular damage [120]. Alcohol interferes with IGF-I receptors and disrupts cell division, which can disrupt the survival of cells and therefore increase inappropriate apoptosis [41]. This prevents the normal function and production of central nervous system cells that are ultimately affected by increased oxidative stress and mitochondrial damage. More research investigating the therapeutic potential of stimulants to natural growth factors that would otherwise be inhibited by alcohol exposure could alleviate the deleterious effects of alcohol on cells during the early development stages.

Effects on Glial Cells

Microglia

Overactivation of neuronal apoptosis by microglia may pose a potential mechanism for decreased brain weight in newborns exposed to alcohol *in utero* [121]. This abnormal neuronal cell loss in the brain due to alcohol exposure during fetal development likely causes irreversible damage on the developing brain. Microglia are the major phagocytic cells (consumers of foreign material) of the central nervous system (CNS) and have been shown to have a significant role in neuronal development in mice by constantly monitoring functional environments of synapses and synaptic pruning [122, 123]. During late fetal and postnatal development, microglia strictly control regulated-cell death of excess neuronal cells via apoptosis throughout the brain of mice, including the hippocampus and cerebellum [124, 125]. Normal microglia function is optimized to minimize inflammation following neuronal apoptosis [124], however, in the presence of ethanol, neuronal apoptosis by microglial cells triggers the release of pro-inflammatory cytokines, including TNF-β, IL-1β, and nitric oxide in third-trimester equivalent postnatal mice [126]. The toll-like

receptor 4 (TLR4) pathway, which normally is important in synaptogenesis and synaptic regulation, is one important mediator of this pro-inflammatory cytokine response and the increased apoptosis due to ethanol exposure [127]. A recent study found that TLR4 knockout (KO) mice did not experience increased apoptosis when their neurons were cultured with ethanol, compared to control Wild-type (WT) mice [126]. Further, when compared to WT mice given an equal exposure of ethanol prenatally, KO mice showed significantly lower levels of microglial activation, cytokine release, and synaptic alterations, as well as reduced memory and anxiety impairments [127]. Altogether, this points to the TLR4 pathway being a significant mediator in microglial dysfunction due to prenatal alcohol exposure, resulting in physical malformation of the brain.

Oligodendrocytes

Cognitive and sensory processing deficits are common symptoms in patients with FAS [128]. A 2007 study found clear oculomotor dysfunction and decreased overall executive function in a cohort of children with FASD [129]. Chief among sensory dysfunction in FASD is a high frequency of different visual deficits in FASD patients, including deficits in visual acuity, visual spatial memory, and visual processing (see Chap. 11 for more detail) [130, 131]. Damage from prenatal alcohol exposure targeting oligodendrocytes and oligodendrocyte precursor cells (OPCs) has clear, lasting effects for FASD patients. Oligodendrocytes play an important role in neuronal development and protection, producing the myelin sheath that lines CNS neurons [132]. OPCs myelinate new axons and differentiate into mature oligodendrocytes throughout fetal development and into adulthood, which allows the CNS to actively remodel its infrastructure as needed [133]. This constant development and restructuring suggest that early fetal development can leave a lasting impact on future brain development. Although myelination can still occur following ethanol exposure *in utero*, the existing damage may be irreversible, as observed in FASD patients [134]. Early imaging studies support this, with one study showing decreased myelination and generalized hypoplasia throughout white matter regions of the brain in patients with severe prenatal alcohol exposure [135]. In third-trimester equivalent mice exposed to ethanol vapor, there was a 58% decrease of mature oligodendrocytes and 75% decrease of OPCs in white matter regions of the brain [136]. Because myelination has an essential role in cognition and healthy brain function [137], prenatal alcohol exposure's interaction with oligodendrocytes and OPCs is a likely contributor to the cognitive deficits common in FASD. Further, demyelination of the optic nerve was observed in rats exposed prenatally to alcohol [138], while a clinical study uncovered ~50% prevalence of optic nerve hypoplasia in a group of children in Sweden born to parents with alcohol use disorder [139]. Such high frequency of oligodendrocyte dysfunction poses a possible mechanism for sensory processing deficits in FASD. While the role of oligodendrocytes in FASD symptoms is not yet entirely understood, current evidence strongly points to its strong negative impact on changes in overall executive function seen commonly in patients with FASD.

Astrocytes

Marked neuroinflammation, decreased brain density and cortical development, and even microcephaly are all associated with prenatal alcohol exposure [140]. Astrocytes have an important function of maintaining the integrity of the central nervous system by maintaining homeostasis, which include maintaining the blood–brain barrier, providing neuronal support via reuptake of necessary ions, and many other homeostatic functions [141]. The unique position of astrocytes in many essential brain functions makes them especially susceptible to damage from prenatal alcohol exposure. Alcohol exposure in astrocytes triggers a pro-inflammatory state through activation of the TLR4/IL-1R pathway (14). Ethanol-induced activation of these pathways has been demonstrated to release pro-inflammatory cytokines TNF-β, IL-1β, and COX-2 in cerebral cortex cell cultures of third-trimester-equivalent mice [142]. This is not dissimilar from the role of microglia discussed earlier, and in fact, astrocytes and microglia may have some direct interactions. Microglia may produce ROS following prenatal alcohol exposure in response to TLR4 pathway activation, which may serve as a mechanism for interaction with astrocytes [126]. One study in tadpoles exposed to ethanol prenatally found ROS inhibited Pax6 gene expression [143]. When catalase, the enzyme that breaks down H_2O_2, was overexpressed, it helped to limit microcephaly in the tadpoles [143]. While more research into this systemic pathway is necessary, the crosstalk between astrocytes and microglia likely contributes to a pro-inflammatory state that may negatively affect neurodevelopment.

Alcohol is also linked with decreased proliferation and differentiation of astrocytes, specifically inhibiting muscarinic-induced proliferation of astrocytes in cell cultures of gestational day 21 rat cortices [134]. Furthermore, alcohol decreases the number and density of astrocytes due to apoptosis in the cerebrum and somatosensory cortex of adolescent-equivalent rat pups exposed to ethanol during the late first and second trimester [99]. These effects contribute to the decreased brain density and cortical development attributed with prenatal alcohol exposure. Loss of astrocytes, autoimmune destruction, and astrocyte integrity due to alcohol exposure therefore contribute to the already agitated neuroimmune system in FASD.

Neurotransmitter Signaling

Norepinephrine/Epinephrine

Children and adolescents with FASD are at a significantly higher risk for experiencing developmental delays, mental illness, and substance use disorder [144]. Growing evidence has strongly linked ADHD and FASD as comorbidities in affected populations, with one study reporting that >60% of patients qualified for comorbid diagnoses based on cognitive and emotional tests [145]. Similarly, substance use disorder is more common in those diagnosed with FASD, with one estimate placing the

frequency at nearly 40% [146]. The numerous effects of prenatal alcohol exposure on adrenergic systems in the CNS have vast consequences, but the full scope of disturbance is not yet fully understood. Norepinephrine (NE) and epinephrine are catecholamines, produced as hormones by the adrenal glands, and also released as excitatory neurotransmitters in both the central and peripheral sympathetic nervous systems. NE is synthesized by the CNS in the locus coeruleus. NE is an important neuromodulator with a variety of functions—arousal, memory, attention, emotions, and other diverse functions—depending on the brain region in which it acts [147, 148]. During gestation and postnatal development, NE also contributes to synaptic plasticity in a variety of ways. One study in neonatal rats (a model for third-trimester-equivalent exposure in human pregnancy) found that NE acted as a potent neurotrophic factor promoting development of adrenergic pyramidal neurons in the cerebral cortex [149]. NE is also believed to have an important role in infant attachment and sensory processing in the olfactory bulb [150, 151]. Ethanol exposure during pregnancy has been strongly linked to decreased NE levels, particularly within the hypothalamus and corpus striatum of prenatal pups [152]. Further, ethanol exposure during fetal development decreases the stability and number of NE-producing locus coeruleus neurons [153]. Because of the importance of the striatum and hypothalamus in emotional regulation and the brain reward circuit system, deficiencies in NE may mediate increased rates of substance use disorder and other reward seeking behaviors common in FASD. Despite these NE deficiencies, mice exposed to ethanol prenatally (equivalent to the first and second trimester periods of human pregnancy) exhibited marked increase in NE transporters in the striatum, which may be a homeostatic response to decreased NE levels being produced [154]. As NE transporters are a point of interest in ADHD, increases in transporters due to prenatal alcohol exposure may be tied to greater onset of ADHD symptoms. NE's strong relation to attention, arousal, and other executive functions may further underly characteristic symptoms of FASD, however this research is yet underdeveloped.

Dopamine

When measuring executive function using a variety of cognitive tests, children prenatally exposed to alcohol or diagnosed with FAS test significantly lower than alcohol-free controls [155]. With evidence of decreased levels of dopamine in the brains of rat pups exposed to ethanol prenatally [152], dopamine deficits may contribute to common cognitive phenotypes seen in FASD, including increased rates of ADHD and other learning disabilities, as well as substance abuse disorders [144]. Dopamine is an excitatory and inhibitory catecholamine neurotransmitter of the central nervous system. Dopamine has a vast array of functions in the CNS, including motor control, higher cognition, reward systems, and working memory [156]. Dopaminergic neurons originating in the basal ganglia project throughout the brain to areas such as the limbic system and prefrontal cortex. Following normal prefrontal cortex development, dopamine acts as a neuromodulator that enables higher

level processing, such as working memory and decision making [157]. In neonatal, third-trimester-equivalent rat pups, prenatal alcohol exposure reduced dendritic spine density and altered dendritic organization in layers II and III of the medial prefrontal cortices [158]. Given the known importance of the prefrontal cortex in decision-making and impulse control functions, these findings likely contribute to the frequent comorbidity of FASD and ADHD [145]. The interaction between dopaminergic neurons and prenatal alcohol exposure has been further investigated in adult rhesus monkeys, that were either exposed to alcohol a) continuously throughout pregnancy, or b) only during the first-early second trimester of pregnancy. Both unique cohorts of rhesus monkeys demonstrated decreased function of their dopamine systems following moderate-to-high levels of ethanol exposure *in utero* [159]. Another study uncovered similarly impaired striatal dopamine system efficiency in rats exposed to ethanol *in utero* [160]. Rats exposed to ethanol prenatally (throughout the first and second trimester- equivalent periods of human pregnancy) exhibited significantly lower levels of dopamine in the striatum. The striatum's reliance on dopamine to regulate many cognitive functions including working memory [161], outlines how serious dopamine deficiencies may impact critical early developmental periods in patients with FASD. Overall, FASD and dopamine are heavily intertwined, and dopaminergic systems are thus susceptible to damage caused by prenatal ethanol exposure. It is likely these effects contribute to the many cognitive symptoms of FASD.

Gamma-Aminobutyric Acid (GABA)

GABA is the main inhibitory neurotransmitter of the adult CNS. Prenatal alcohol exposure likely affects the GABA system of the CNS at many different stages of development, thereby contributing to long-lasting consequences for FASD patients. When heavy prenatal alcohol exposure coincides with GABAergic interneuron development, severe prenatal brain malformations, such as hydrocephalus and then displacement of brain layers, as well as low birth weight can occur [162]. Further, interactions between alcohol and developing GABA systems regulate the development of fine motor skills, with one study finding a significant association between patients with FASD and underdeveloped motor skills, including coordination and balance [163]. In the developing CNS, GABA acts as an autocrine neurotrophic factor, promoting neuronal proliferation/growth in early embryonic chicks [164]. One subtype of GABAergic neurons are uniquely specialized interneurons that begin migrating tangentially and radially from the medial ganglionic eminences during the late-first trimester, and continue thereafter as needed throughout the telencephalon of the brain [165]. This helps to create complex formations of GABA interneurons in the developing brain [166]. In second-trimester equivalent rats exposed to ethanol, there is a marked decrease in neuronal cell proliferation in the medial ganglionic eminence and an increase in premature tangential GABA neuronal migration from the medial ganglionic eminence throughout different ventricular zones [167].

While multiple cells function using GABA neurotransmitters, Purkinje cells, GABAergic projection neurons found in the cerebellum, may be especially vulnerable to damage due to prenatal alcohol exposure [168]. Purkinje cells regulate complex motor function through inhibitory signaling in the CNS. These cells have been shown to be extremely vulnerable to ethanol exposure, with documented and pronounced apoptosis due to a single exposure of ethanol in postnatal day 4 rats, which coincides with the third trimester of human pregnancies [169]. Another study in macaque monkeys suggests that ethanol exposure in the first trimester equivalent or throughout the entire gestational period decreases GABA neuronal density in the somatosensory cortex of the brain, suggesting a more generalized inhibition of GABAergic function [95]. These differences in somatosensory cortex composition may be a result of premature tangential migration discussed earlier. GABA systems in the CNS are a clear target of alcohol's teratogenic effects from conception to birth.

Serotonin

Persons with a diagnosis of FASD are at higher risk for developing disorders of mood and affect, including anxiety, depression, ADHD, substance use disorder, and suicidal ideation [144]. FASD in males is linked with a ~20-fold increase in suicidal ideations and attempted suicide compared to the national average [170]. One postmortem study in humans has found that individuals who committed suicide had marked decreases in Serotonin (5-HT) levels compared to the general population [171]. In the context of FASD, decreased 5-HT levels may be a possible mechanism that leads to increased severe mental illness. 5-HT has been recognized as an important inhibitory neurotransmitter for numerous higher level brain functions, including homeostatic maintenance and behavioral control [172]. Clusters of 5-HT neurons in midbrain and brainstem raphe nuclei, project widely - anteriorly into forebrain and posteriorly into spinal cord - to influence perceptual, cognitive, and affective responses in the mature adult brain [173]. However, during early fetal development, 5-HT, like other neurotransmitters, acts as a neurotrophic factor, and in an explant culture model was shown to promote proliferation, growth, and differentiation of new serotonergic cortical neurons [174]. This 5-HT function is especially important in FASD, because serotonergic neurons are known to begin differentiating and proliferating heavily in the brain stem by the mid-first trimester in human fetuses [175]. The first trimester is also the period of pregnancy with the highest frequency of alcohol use. One study in the United States found that almost 50% of participants consumed alcohol at some point during their early pregnancy [176]. Chronic prenatal alcohol exposure has also been shown to decrease serotonin levels globally in third-trimester rats [177] and diminishes proliferation, maturation, and migration of serotonergic neurons in the forebrain of late prenatally exposed mice [178]. It is likely that these deficits persist well after birth, as a study examining adolescent mice exposed to ethanol during the second trimester reported chronic deficits in 5-HT neurons in the dorsal and medial raphe nuclei due to an increase of caspase-3 [179, 180]. Serotonin deficiency has been strongly implicated in

disorders of affect such as depression and anxiety [181] and poses a potential mechanism for the frequency of developing mental illness in FASD. Consequently, serotonergic circuit dysfunction is predicted to constitute an important component of the neurobehavioral disabilities associated with FASD.

Immune Function

Compromised immune function and predisposition to infection have long been reported in the FASD population [182, 183] and have been recapitulated in animal studies [184–186]. Underlying causes of this include:

- *Disruption of normal thymic development from neural crest cells following prenatal alcohol exposure* [187], *which lead to abnormal proportions of T cell subsets* [188, 189] *and T cell dysfunction* [106, 190, 191].
- *Altered interleukin-2 (IL-2)/IL-2 receptor interactions* [106, 192, 193].
- *Perturbed norepinephrine/β-adrenoreceptors regulation of immune cell populations in lymphoid organs* [194].

Innate Immunity

Innate immunity entails nonspecific defense mechanisms that come into play immediately or within hours of an antigen's appearance in the body, and consists of physical, chemical, and cellular defenses against pathogens. In particular, natural killer (NK) cells and myeloid lineage cells (e.g., monocytes, macrophage, dendritic cells, and neutrophils) have a role in innate immunity and are impacted by prenatal alcohol exposure. Basal increases in NK and myeloid lineage cells were observed in secondary lymphoid organs of rats prenatally exposed to ethanol [191]. Furthermore, there is decreased NK cell cytotoxic activity [195] and increased pro-inflammatory myeloid cell-derived cytokines (e.g., TNF and IL-1β) in prenatally-exposed adult rats [196]. This means innate immune cell function is perturbed, in addition to overall population numbers, further disrupting the normally precise balance that is required in immune system regulation. The consequences of this disruption may manifest as adult-onset neuropathic pain [191] or increased risk of respiratory infection in newborns. Specifically, a decrease in the antioxidant glutathione as a result of *in utero* exposure to ethanol leads to impaired differentiation and phagocytic activity of alveolar macrophages in the lungs of rodents [197–199]. This increases subsequent risk for experimentally-induced pneumonia in newborn pups [200]. Potentially exacerbating this alveolar macrophage dysfunction in the lung is a decrease in the surfactant proteins (SP) SP-A and SP-D observed in sheep exposed to ethanol *in utero* [201, 202]. These proteins are essential mediators of the local immune response in the lung, modulating dendritic and T cell function and facilitating removal of pathogens by alveolar macrophages [203]. Taken together, impairment of innate immunity in FASD may contribute to a predisposition for infection.

Adaptive Immunity

In addition to deficits in innate immunity, there is also dysfunction in adaptive immunity. Adaptive immunity, also known as acquired immunity, mobilizes after innate immunity has proven insufficient to remove invading pathogens and consists of humoral (antibody-mediated) and cell-mediated defenses that adapt to the specific pathogen, enhancing the immunological response. In particular, B cell and T cell lineages have proven sensitive to *in utero* ethanol exposure. Impaired B cell function is typically associated with recurrent infections by encapsulated and pyrogenic bacteria, while impaired T cell function is usually associated with recurrent opportunistic infections and viral and fungal infections. All of these types of recurrent infections are common in FASD children [182], indicating that both lymphocyte lineages are impacted.

B Cell Lineage

Reduced numbers of splenic and bone marrow B cells were found in postnatal mice following prenatal alcohol exposure, and this reduction persisted until adolescence [204, 205]. Moreover, isolated B cells showed a weakened proliferative response to lipopolysaccharide (LPS; a bacterial cell wall component) [204]. Following intrauterine alcohol exposure, impaired differentiation into mature B cells has been demonstrated in B lineage cells from liver [206, 207] and in oligoclonal-neonatal-progenitor (ONP) cells [208, 209], which are capable of differentiating into B lymphocytes depending on the cytokines to which they are exposed. Specifically, the ONP cells isolated from prenatal alcohol exposure newborn mice had a greatly reduced capacity to commit to the B cell lineage. Additional investigations have demonstrated that alcohol affects ONP cell differentiation into B cell lineage by downregulating the expression of several transcription factors and cytokine receptors [210].

T Cell Lineage

Prenatal alcohol exposure also impacts thymocyte development. Rodent studies have shown delayed thymic development [211], decreased number of thymocytes, and reduced proliferation response to stimulation by thymocytes isolated from late-second trimester mouse fetuses [212]. This alcohol-associated suppression of proliferative response and cell numbers of thymocytes has been shown to persist through childhood and begin to return to normal or elevated levels by adolescence [189, 190, 213–215]. Although this proliferative capacity may recover by adolescence, the lasting consequences of a perturbed developmental environment on the T cell lineage are persistent, with lasting T cell dysfunction [106, 190, 191] and alterations in T cell numbers [188, 189]. This lasting dysregulation contributes to increased susceptibility to infections across the lifespan [182, 184, 185] and other

autoimmune/inflammatory related diseases, such as adjuvant-induced arthritis (a model for rheumatoid arthritis) [216] and adult-onset neuropathic pain resulting from a predisposition for allodynia in FASD individuals [191].

Maternal and Fetal Cytokines as Biomarkers for FASD

Because of the distinct impact alcohol has on the immune system in both adults and in utero, recent studies have examined whether there exists specific immunological biomarkers of FASD. Of primary focus have been cytokines, which are small peptide molecules that are crucial in cell-cell signaling of the immune system. Cytokines are present in all tissues and, most importantly, in circulation, making them readily measurable after a simple blood draw. Prenatal alcohol exposure influences cytokine profiles of both the pregnant individual and child after birth. This provides a unique opportunity to define unique cytokine profiles in pregnant individuals and children that can be used to identify those children at increased risk of neurodevelopmental delays, and to provide a supplemental diagnostic tool to help physicians diagnose FASD [217–219].

Non-Protein-Coding RNAs

Many of the teratogenic consequences of prenatal alcohol exposure may be mediated through non-protein-coding RNAs (ncRNAs), which are distinct from messenger RNA (mRNA) in that they are not translated into protein. They perform a wide variety of regulatory functions, modulating mRNA and protein levels via complex signaling networks of which they are a part. Moreover, these RNA and protein networks play a crucial role in developmental processes [220, 221]. The specific ncRNAs that are key components of these networks are sensitive to environmental changes, such as prenatal alcohol exposure, as discussed below.

microRNAs

MicroRNAs (miRNAs) are a diverse and plentiful class of short ncRNAs (19–25 nucleotides) with tissue-specific expression patterns that vary temporally and spatially [222] and inhibit protein translation by targeting RNA-induced silencing complex (RISC) to the 3′ untranslated region (UTR) of mRNA [223]. miRNAs can be secreted as paracrine or endocrine signals capable of choreographing gene expression and function in recipient cells [224, 225]. Through these mechanisms of action, miRNAs regulate developmental timing and pattern formation, promoting the rapid clearance of transcripts as cells transition from one state to another during development, and fine-tuning gene expression [226].

Research has shown that miRNAs are sensitive to prenatal alcohol exposure. One study of miRNAs was performed using cultured neuroepithelial cells isolated from the mouse fetal cerebral cortex, and revealed several differentially expressed miRNAs in response to ethanol treatment, including the suppression of miR-9, a crucial regulator of neurogenesis [94]. Moreover, genetic inhibition of miR-9 in zebrafish and mice results in morphological features associated with FASD, such as microcephaly [227, 228] and, for zebrafish, results in the same juvenile swimming phenotype as that of alcohol-treated animals [227, 229]. Another study found similar results in primary cultures of cerebellar granule neurons isolated from neonatal mice, identifying miR-9, miR-29a, and miR-29b as decreased after ethanol exposure [230]. This study also showed that miR-29b may mediate ethanol-induced apoptosis during the period of cerebellar sensitivity to alcohol exposure. Moreover, in primary neuronal cells from fetal mice, chronic intermittent ethanol exposure resulted in widespread alterations in miRNA expression profiles [231]. Importantly, this study demonstrated that even after ethanol withdrawal, this altered expression of miRNAs persisted, revealing that alcohol exposure can permanently reprogram the miRNome. Similarly, widespread alterations of miRNA were identified in the brains of adult mice exposed to ethanol prenatally [232], supporting the idea that prenatal alcohol exposure causes long-term reprogramming of ncRNA networks which result in persistent teratogenicity.

Excitingly, circulating miRNAs are being interrogated for use as potential diagnostic biomarkers of FASD because of consistent alterations in the patterns of miRNAs perturbed by developmental ethanol exposure. The first study to address this idea profiled plasma miRNAs in pregnant ewes and newborn lambs exposed to alcohol, identifying miR-9, miR-15b, miR-19b, and miR-20a [233]. Research has progressed to human studies with populations based in Ukraine [92] and South Africa [234], identifying specific miRNA panels in children prenatally exposed to alcohol. Subsequent analyses have identified infant sex as an important factor for consideration when developing more accurate miRNA biomarker panels for diagnosis [235]. As this research progresses, sensitive miRNA panels may be another useful tool in aiding physicians to provide a diagnosis of FASD to their patients.

Long Noncoding RNAs

Long noncoding RNAs (lncRNAs) are >200 nucleotides in size and lack the potential to code for a protein >100 amino acids [236]. lncRNAs play an important role in development, as shown in the developing foregut and lungs [237], brain [238], and adipose [239], and in osteogenic differentiation of mesenchymal stem cells [240]. lncRNAs may carry out their developmental role by binding chromatin modifying complexes such as the histone methyltransferase G9a or the polycomb repressive complex (PRC)2 [241–243]. By interacting with these protein complexes, lncRNAs guide them to either modify or “read” chromatin in the promotion of pluripotency and repression of differentiation signals [244, 245].

The impact of developmental alcohol exposure on lncRNAs remains poorly understood, but is a promising avenue of research for providing epigenetic control during development. To date, there are three studies that directly measure the consequences of developmental alcohol exposure on lncRNA expression. The first identified is linc1354, which is associated with neural stem cell differentiation and interacts with PRC2, and is decreased in fetal mouse neurospheres following ethanol exposure [246]. This decrease in linc1354 may impact downstream targets of PRC2 that are crucial in normal developmental gene regulatory networks, resulting in abnormal differentiation in the fetal brain. A second study identified the lncRNA Oct4 pseudogene on mouse chromosome 9 (*m*Oct4pg9) as being increased following ethanol exposure in fetal mouse neurospheres [247]. Moreover, this study determined that *m*Oct4pg9 is associated with increased cell proliferation and maturation, potentially contributing to the pro-maturation effects of ethanol exposure that result in the loss of neural stem cells and subsequent decrease in brain growth. A third study showed suppression of the lncRNA *Xist* in female mouse fetuses exposed *in utero* to ethanol [248]. Normally, *Xist*, which is located on the X chromosome, inactivates the second X chromosome present in female cells (XX), modulating expression of X-linked genes so that a single copy is expressed in females, similarly to how males (XY) only express one copy. However, in this study, prenatal alcohol exposure resulted in decrease of *Xist* and loss of X-inactivation, with subsequent changes in X-linked genes and gene regulatory networks during neural development. Altogether, these studies support the idea that lncRNA are sensitive to the teratogenic consequences of developmental alcohol exposure, consequently perturbing neurogenesis and organogenesis.

Circular RNAs

Another type of ncRNA that may mediate the consequences of prenatal alcohol exposure is circular RNAs (circRNAs). circRNAs are ncRNAs greater than 200 nucleotides that have circularized by covalently bonding a 3′ downstream donor to a 5′ upstream splice acceptor in a process referred to as “backsplicing” [249]. circRNAs may play a crucial role in ncRNA networks by acting as sponges for miRNAs, as they often contain multiple in-tandem miRNA binding elements, allowing for de-repression of miRNA target genes [250, 251]. Furthermore, circRNAs have been shown to play a role in development in various organs of the body, such as the heart, lungs, and brain, demonstrating spatio-temporal dynamics as development of the embryo progresses [252–254].

Normally, circRNAs are a part of the homeostasis of RNA networks that are finely balanced during development. However, the balance of RNA networks may be disrupted if a teratogenic exposure occurs, a growing area of research in the FASD field with only one study to date. This study found that prenatal alcohol exposure alters circRNA expression in whole brains of second trimester-equivalent mice in a sex-specific manner [255]. The study also found that prenatal alcohol exposed males and females had similarly altered expression of protein-coding mRNAs when

compared to their control counterparts. Interestingly, circRNA expression was altered in a sex-specific manner. Prenatal alcohol exposed females had a specific set of upregulated and downregulated circRNAs, and this specific set of circRNAs did not overlap with those altered in prenatal alcohol exposed males. This suggests that though altered mRNA expression may be shared between males and females after prenatal alcohol exposure, the regulatory networks by which they are achieved are sex-dependent. This is an important factor to consider when designing therapeutics targeting similar regulatory networks, because such therapeutics may need to be sex specific.

More studies on the role of circRNAs in the etiology of FASD are needed, particularly because of the significant potential for circRNAs to serve as therapeutic targets. For instance, circRNAs packaged in nanoparticles or extracellular vesicles (EVs) have been used as a therapy in animal models of disease [256–259]. Once inside their target cell, the circRNAs are thought to act as a miRNA sponge, helping to balance the RNA network that was disrupted by a disease state. Alternatively, in diseases characterized by high levels of circRNAs, these circRNAs have been targeted by nanoparticle and EV packaged short interfering RNA (siRNA) or short hairpin RNA (shRNA) that target specific circRNAs for destruction, returning levels to closer to normal [260–262]. This may be a promising therapeutic avenue for complex disorders that result in the dysregulation of entire gene networks.

Teratogenic Consequences of Ethanol-Induced Epigenetic Modifications

Epigenetic modifications (DNA changes that impact gene expression without altering the DNA sequence) are closely associated with early brain development [263], cardiogenesis [264], immune system development [265], and organogenesis in general. Alterations to the normal progression of these modifications, which have been shown to occur following prenatal alcohol exposure, can lead to lasting neurodevelopmental and behavioral consequences [266], congenital heart disease [264], and autoimmune disorders [267] (Fig. 5.4).

DNA Methylation

Ethanol-induced changes in DNA methylation status have several outcomes. On a cellular level, ethanol exposure alters the natural progression of DNA methylation status in rat neural stem cells [268]. This resulted in changes in expression of genes associated with differentiation and in turn, changes in phenotypes, such as delayed neuronal formation, migration, and growth processes. The exact timing of prenatal alcohol exposure during pregnancy (i.e., either first, second, or third trimester exposure) can uniquely perturb DNA methylation status, with each exposure window resulting in its own set of genes altered by ethanol [269]. These methylation

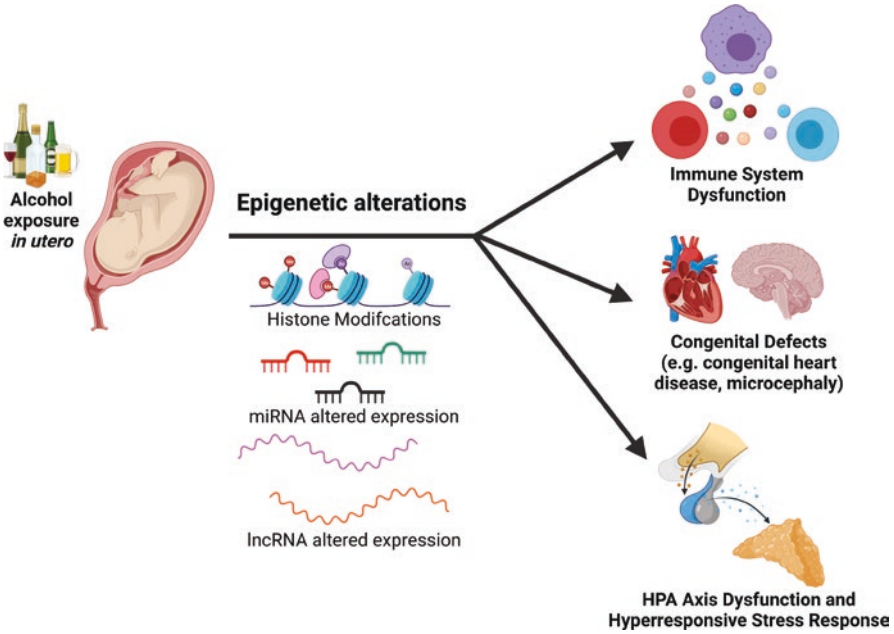

Fig. 5.4 The effects of prenatal alcohol exposure on epigenetic profiles and health outcomes in offspring. Prenatal alcohol exposure induces multiple forms of epigenetic changes, including histone modifications and shifts in expressions of micro(mi)RNA and long noncoding (lnc) RNA. These alcohol-induced alterations have been associated with poor physical and behavioral health outcomes in exposed offspring

perturbations last into adulthood in mice, indicating the life-long consequences of these early life, prenatal alcohol exposure-sensitive changes. While the exact set of changed genes is unique to each exposure window, the overall prenatal alcohol exposure-sensitive pathways and phenotypes overlap (e.g., synaptogenesis, apoptosis, cellular identity, cell–cell adhesion, and signaling), indicating that these developmental pathways are vulnerable to prenatal alcohol exposure throughout pregnancy.

Furthermore, the lasting impact of ethanol exposure on cellular phenotypes also manifests on a larger scale in behavioral phenotypes that last into adulthood. One study found that increased anxiety-like behavior in adult prenatal alcohol exposure rats was associated with increased expression of DNA methyltransferases in the hippocampus [270], an outcome that would presumably lead to increased DNA methylation, and consequently, heterochromatin formation. However, another study documented decreased expression of mRNA transcripts for methyl CpG binding protein 2 (MeCP2, a protein which binds to methylated regions of chromatin to prevent transcription) in the cortex and striatum of adult rodents prenatally exposed to alcohol. Moreover, this decrease in MeCP2 mRNA was associated with the FASD-like phenotypes of increased impulsivity, hyperactivity, and inattention [154]. Altogether, these studies show that prenatal alcohol exposure's effects on the

epigenetic landscape are complex, and modifications that occur during development can persist throughout the lifespan.

These lasting methylation changes implicate a possibility of defining an epigenetic profile that serves as a biomarker for prenatal alcohol exposure [271–273]. Genes identified in these studies are implicated in a number of FASD phenotypes, such as cognitive function, anxiety, attention deficit hyperactivity disorder, and mood disorders, revealing a link between altered DNA methylation and observed FASD phenotypes and symptoms. This means that a clearly defined epigenetic profile of prenatal alcohol exposure may potentially serve as a diagnostic tool for identifying individuals with FASDs. One such study has used buccal swabs from children to determine individual DNA methylation profiles [274]. While the study did successfully identify a potential diagnostic profile that could span two different cohorts, the study was limited due to small sample size and a lack of demographic diversity. However, there is a strong potential to develop a more universal diagnostic profile with further investigation.

Histone Modifications

Prenatal alcohol exposure also affects the epigenome via histone modifications. These modifications can either be gene-repressive or gene-activating, and this classification is specific to the histone residue and the precise modification. This means that while there is no generalized rule for histone methylation, the histone methylation code is specific in terms of site and degree of modification for whether gene expression is increased or decreased.

The most studied histone modification after prenatal alcohol exposure is histone H3 lysine 9 acetylation (H3K9ac), which is associated with gene-activation [275, 276]. Of particular interest for prenatal alcohol exposure research has been the role of altered H3K9ac in cardiac development, as prenatal alcohol exposure increases the rate of heart defects [277–280]. This higher rate of congenital heart defects may, in part, be attributed to increased H3K9 acetylation. Studies using culture models of cardiac progenitor cells demonstrate that increased H3K9 acetylation results in increased expression of key cardiac development genes [281, 282]. When continued in a mouse model, increased histone acetyltransferase (HAT) expression and activity coincided with abnormal heart development in the fetal mice, resulting in congenital heart defects similar to those associated with FASDs [283].

Additionally, the effect of prenatal alcohol exposure on histone methylation has been a large focus in prenatal alcohol exposure research. Ethanol exposure in neonatal mouse pups, equivalent to the late pregnancy period in humans, increased methyltransferase expression and activity led to increased dimethylation of histones in the brain [284, 285]. This finding was supported by another study showing a persistent increase in histone dimethylation up to gestational day 17 (GD17) in fetal mice previously exposed to ethanol on GD7 [286]. This increase in histone dimethylation was consistently observed in prenatal alcohol exposed pups with facial

dysmorphologies, but largely absent in prenatal alcohol exposed pups without facial dysmorphologies, and was found to robustly occur on genetic loci for a panel of genes associated with normal progression of neural development. Increases in histone dimethylation have also been documented in 2-month-old male rats, i.e., early adulthood, along with additional prenatal alcohol exposure-induced histone modifications, in the arcuate nucleus of the hypothalamus [287, 288]. The lasting consequence was decreased expression of the proopiomelanocortin (Pomc) gene transcript, a precursor for adrenocorticotrophic hormone among other hormones. Interestingly, the inhibition of Pomc was counterintuitively associated with a hyperresponsive adrenal gland, suggesting destabilization of an important neuroendocrine circuit for stress management. In contrast to the observed methylation of the POMC gene locus, other studies have also shown a global decrease in methylation, which is usually attributed to decreased availability of the methyl donor S-adenosylmethionine. One study has shown that an ethanol-induced decrease in histone methylation alters the normal progression of gene expression during neurogenesis [246] while another demonstrated suppression of osteogenesis and adipogenesis in mesenchymal stem cells after ethanol exposure [289]. Taken together, global histone modifications may contribute, in part, to the lifelong repercussions of prenatal alcohol exposure.

Potential Therapeutics Targeting Prenatal Alcohol Exposure-Induced Histone Modifications

Identifying histone modifications as a mediator of the lasting consequences of prenatal alcohol exposure raises the exciting possibility of potential pharmacological and nutritional interventions. There are a number of natural HAT inhibitors, such as turmeric and anacardic acid, that may be able to mitigate the effects of prenatal alcohol exposure. One study demonstrated that use of curcumin, a compound found in turmeric, can reverse increased histone acetylation in the caspase-3 and caspase-8 promoters in cardiocytes, reducing apoptosis after ethanol exposure [290]. Additionally, another group of researchers treated pregnant mice simultaneously with anacardic acid and ethanol and found that co-administration of anacardic acid, a phenolic lipid derived from cashew nut shells, significantly reduced ethanol-induced hyperacetylation of histones in cardiac tissues [281]. The effects of prenatal alcohol exposure could also be mitigated through choline supplementation to increase histone and DNA methylation, as choline can contribute to increased levels of methionine. Choline supplementation has been shown to normalized histone and DNA methylation, *Pomc* expression, and corticosterone levels in offspring of pregnant rats when supplemented concurrently with ethanol exposure [287]. Choline supplementation is currently under clinical investigation as a therapeutic intervention for prenatal alcohol exposure consequences on neurobehavior and growth [291, 292].

Transgenerational Consequences of Prenatal Alcohol Exposure via Genomic Imprinting

Epigenetic modifications extend beyond the direct effects of prenatal alcohol exposure during pregnancy by perturbing genomic imprinting, a form of epigenetic inheritance that causes genes to be expressed in a parent-of-origin-specific manner, prior to conception. This means that preconception ethanol exposure in germ cells (i.e., sperm and oocytes) can contribute to phenotypes typically associated with prenatal alcohol exposure or FASDs. For example, one multigenerational study traced prenatal alcohol exposure-induced increases in *Pomc* promoter methylation through the male germline [288]. Researchers found decreased *Pomc* expression in the hypothalamus and increased adrenocorticotropic hormone (ACTH) and corticosterone levels in the plasma of both male and female offspring of first generation (F1) progeny, but this pattern only persisted in males of the male prenatal alcohol exposure germline in F2 and F3 progeny, pointing to male germline transmission of increased methylation of the *Pomc* promoter. This same group later demonstrated similar findings for interferon-γ (IFN-γ), a cytokine known to regulate both innate and adaptive immunity. Increased methylation for *Ifn-γ* gene promoter and subsequent decrease in *Ifn-γ* mRNA were identified in both F1 males and females [293]. However, this methylation and expression pattern was continued only in the male germline to the F3 generation. The implication of these data is that prenatal alcohol exposure can directly affect the developing fetus at the time of ethanol exposure, but can also impact future progeny of that fetus, thus affecting families across multiple generations.

Looking Forward: Identification of Risk and Resilience Factors

In this chapter, we have addressed a multitude of targeted research avenues that can inform the advancement of treatments for individuals with FASD. As a developmental disorder, FASD poses unique challenges for healthcare professionals and clinicians, including the absence of a definitive diagnostic test to uncover prenatal alcohol exposure in offspring, and social stigmatization which limits accurate self-reports of drinking by pregnant individuals. When considering the complex interactions of exposure variables (including timing during gestation and pattern/repetition of exposure) and subjects (sex, age, species, etc.) on symptom expression, it is perhaps unsurprising that debate exists among medical professionals and the general public about the dangers of alcohol exposure during pregnancy. However, we have aimed to demonstrate through our discussion of alcohol toxicology and teratology, that alcohol alters the fate of numerous intra and intercellular systemic functions that can contribute to the persistent deficits observed in humans with known FASD. By acknowledging alcohol as more than a toxic, poisonous agent, and rather

as a highly variable and widespread cellular teratogen, we believe scientists can better refine their research questions and experiments to directly target biochemical systems underlying FASD symptomology. This includes investigating a range of biological and genetic differences between offspring prenatally exposed to alcohol, and associating these innate phenotypes with symptom expression. Such comparisons can facilitate the isolation of factors that lead a child to be "at risk" or "resilient" to a particular symptom of FASD, and subsequently, can pinpoint specific mechanisms of importance for the development of therapeutic interventions for affected individuals.

Glossary

5-HT (Serotonin) A monoamine transmitter with a variety of functions, including emotional and behavioral regulation.

ACTH (Adrenocorticotropic hormone) A pituitary gland hormone that initiates the production of *cortisol*, which is key to the body's response to stress and infection, as well as regulating blood sugar levels and maintaining blood pressure.

ADHD (Attention deficit hyperactivity disorder) A neurodevelopmental disorder characterized by difficulty with focusing, lack of impulse control, and hyperactivity.

Apoptosis Programmed cell death that can occur as a normal, controlled process to eliminate unwanted or damaged cells, but can also be induced inappropriately with the introduction of a toxin or teratogen.

circRNAs (Circular RNA) Noncoding RNAs greater than 200 nucleotides in size, that form a closed loop from linked 5′ and 3′ termini in a process of exon back-splicing.

CNS (Central nervous system) The part of the nervous system consisting of the brain and spinal cord.

COX-2 (Cyclooxygenase 2) An enzyme involved in the inflammatory response, that converts arachidonic acid to prostaglandins.

Epigenetic(s) Modifications in the expression of a gene, rather than changes in the genetic code itself. Without altering the DNA sequence, external factors can change whether a gene is turned "on"—and can interact with other cellular processes, leading to protein transcription- or turned "off."

GABA (Gamma-aminobutyric acid) An amino acid neurotransmitter, and the primary inhibitory neurotransmitter in the adult CNS.

H_2O_2 (Hydrogen peroxide) A reactive oxygen species, broken down in the body by catalase.

HAT (Histone acetyltransferase) An enzyme that catalyzes acetylation of lysine amino acids on histone complexes.

IBA-1 (Ionized calcium-binding adapter molecule 1) An intracellular protein found in microglial cells.

IL-1 (Interleukin-1 beta) A cytokine produced by macrophages that mediates the pro-inflammatory response.

IL-1R (Interleukin 1 receptor) A cytokine receptor involved in the immune response which binds preferentially to IL1.

IL-2 (Interleukin 2) A pro-inflammatory cytokine mainly involved in the acquired immune response.

Interferon (IFN) A cytokine that is critical for innate and adaptive immunity against viral and some bacterial infections. Named for their ability to "interfere" with viral replication by protecting cells from virus infections, IFNs are produced principally by natural killer (NK) cells.

KO (Knockout) Refers to the use of genetic engineering to inactivate or remove one or more specific genes from an organism. Scientists create knockout organisms to study the impact of removing a gene from an organism, and thus learn about that gene's function.

LncRNAs (Long noncoding RNA) Noncoding RNAs of greater than 200 nucleotides in size. LncRNAs primarily interact with mRNA, DNA, protein, and miRNA and consequently regulate gene expression.

LPS (Lipopolysaccharide) A major surface membrane component present in almost all Gram-negative bacteria. It is essential to both the structural integrity and function of the outer membrane.

MeCP2 (Methyl CpG binding protein 2) A protein that binds to methylated regions of chromatin to prevent transcription.

Microglia Immune cells of the central nervous system, capable of interacting with neurons and non-neuronal cells. They account for ~10% of cells in the brain and are first responders in the brain's response to infections and inflammation.

miR-9 (microRNA 9) A microRNA involved in neurogenesis.

miR-29a (microRNA 29a) A microRNA involved in tumor suppression.

miR-29b (microRNA 29b) A microRNA involved in tumor suppression.

miRNAs (microRNAs) Small, non-protein coding RNA involved in tissue-specific gene expression patterns.

miRNome (Micro RNA genome) A complete set of microRNAs in a genome.

mRNA (Messenger RNA) A type of cellular RNA that carries the genetic information needed to make proteins. mRNA carries the information from DNA, located in the nucleus of the cell, to the cytoplasm, where the proteins are created.

ncRNAs (Non-protein-coding RNAs) A type of cellular RNA that cannot be coded into a protein.

NE (Norepinephrine) An excitatory catecholamine neurotransmitter produced in the CNS and PNS with many associated functions, including attention, memory, and emotional regulation.

NK (Natural killer cell) A cytotoxic lymphocyte and type of white blood cell that is essential to the innate immune response.

OPC (Oligodendrocyte precursor cell) A CNS glial cell that myelinates new axons and develops into mature oligodendrocytes.

Pomc (Proopiomelanocortin) A precursor, or prohormone, to hormones such as adrenocorticotropic hormone and beta-endorphin, which are involved in adrenal function and pain regulation.

PRC2 (Polycomb repressive complex 2) A transcription protein that catalyzes repression of histones

RISC (RNA-induced silencing complex) Protein complex involved in translation silencing.

ROS (Reactive oxygen species) A highly reactive chemical formed from oxygen that acts as a cell signaling molecule.

shRNA (Short hairpin RNA) An artificial RNA molecule that is used to silence target gene expression (gene silencing) through RNA interference.

siRNA (Short/small interfering RNA) A class of double-stranded RNA that interferes with the expression of specific genes by degrading mRNA after transcription, thus preventing translation.

SP-A/SP-D (Surfactant protein A/D) Pulmonary collectin proteins involved in the innate immune response.

TLR4 (Toll-like receptor 4) A transmembrane protein involved in the innate immune response, specifically the Nf-Kb intracellular pathway.

TNF-B (Tumor necrosis factor beta) Also known as lymphotoxin alpha, a cytokine produced by different lymphocytes with various immune functions, including the pro-inflammatory response triggered by microglial cells.

UTR (Untranslated region) A sequence on both ends of an mRNA molecule that is not translated into a protein.

WT (Wild-type) A typical phenotype seen in nature/outside of the laboratory.

XX Sex chromosomes pertaining to female cells.

XY Sex chromosomes pertaining to male cells.

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