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SCHOOL OF MEDICINE

Thesis

THE ROLE OF TERATOGEN EXPOSURE ON NEURAL CREST CELLS IN THE PATHOGENESIS OF FETAL ALCOHOL SPECTRUM DISORDERS

by

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B.Sc., McGill University, 2013

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DEDICATION

To Robert, my uncle and one of my best friends, a man who handles his affliction with grace and dignity. When I think of all that he was unable to achieve because of his disability, I consider all that he was able to achieve despite his disability, and I'm reminded that we all have a contribution to make for society.

THE ROLE OF TERATOGEN EXPOSURE ON NEURAL CREST CELLS IN THE PATHOGENESIS OF FETAL ALCOHOL SPECTRUM DISORDERS

RICHARD BOHLING CAROZZA

ABSTRACT

Maternal consumption of ethanol during pregnancy contributes to a set of pathologies, grouped together as the fetal alcohol spectrum disorders, affecting as many as 5% of live births in the United States annually. Ethanol acts widely in the developing embryo, affecting many tissues, but causing deficits in neuronal and neural crest populations particularly. These deleterious effects cause archetypical craniofacial expression and neurological deficits, including microcephaly and neuronal dysfunction. Severity of symptoms is linked to frequency of maternal alcohol consumption as well as the maximum blood alcohol concentration reached by the mother.

The teratology of ethanol has been widely researched over the last four decades, with the link between the neural crest pathology and the fetal alcohol spectrum phenotype becoming clearer. Animal model studies have managed to replicate many of the symptoms seen in humans afflicted with fetal alcohol spectrum disorders, and have allowed us to elucidate the biochemical mechanisms behind the disease. There is no singular pathway responsible for the fetal alcohol spectrum disorders: over half a dozen models of dysfunction have been identified, and ethanol's ability to react with a series of targets means that more pathways are likely to be discovered.

Current theories regarding the effects of ethanol on the neural crest have implicated apoptosis of the cephalic neural crest, mediated by G-protein coupled receptors, activation of a phospholipase C pathway, and subsequent release of intracellular calcium; perturbations of the actin cytoskeleton leading to migration dysfunction of neural crest cells in the developing neural tube; lack of functional trophic molecules, specifically *Shh*, likely due to dysfunction of the cholesterol biosynthetic pathway; lack of retinoic acid production; oxidative stress, production of reactive oxygen species, and iron dysregulation; and genetics, which seems to confer greater susceptibility and resistance to ethanol in certain individuals. Ultimately, a global model for ethanol's actions on the developing fetus eludes researchers, as do any potential treatments, and more research is required to further elucidate ethanol's teratogenic mechanism.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACAT.....Acetyl-Coenzyme A Acetyltransferase

ATP..... Adenosine Triphosphate

CAM..... Cell Adhesion Molecule

CaMKII..... Ca^{2+}/calmodulin-dependent kinase II

cAMP..... Cyclic Adenosine Monophosphate

CNS..... Central Nervous System

EGF..... Epidermal Growth Factor

FAS..... Fetal Alcohol Syndrome

FASD.....Fetal Alcohol Spectrum Disorders

FSNC.....Facial Skeletogenic Neural Crest

GPCR.....G-Protein Coupled Receptor

HMG-CoA..... 3-Hydroxy-3-Methylglutaryl-Coenzyme A

HPE..... Holoprosencephaly

IP_3..... Inositol Triphosphate

NAC.....N-Acetylcysteine

NADH.....Nicotinamide Adenine Dinucleotide

NCC.....Neural Crest Cell

NT3.....Neurotrophin-3

PAE..... Prenatal Alcohol Exposure

PDGF.....Platelet-Derived Growth Factor

PKA.....Protein Kinase A

PKC..... Protein Kinase C

PLCβ .....Phospholipase Cβ

PME ..... Prechordal Mesendoderm

PrCP ..... Prechordal Plate

ROS.....Reactive Oxygen Species

SHH..... Sonic Hedgehog

SIDS..... Sudden Infant Death Syndrome

SLOS..... Smith-Lemli-Opitz Syndrome

INTRODUCTION

Clinical Presentation of FAS, FASD

Ethanol is the most prevalent human teratogen, and is the largest known contributor to developmental disability (Smith, 2014). The link between prenatal alcohol exposure and negative health outcomes has been known anecdotally for centuries, with the ancient Greeks and Romans prohibiting maternal alcohol consumption. Western medicine often attributed alcohol-associated complications to heredity, and it wasn't until the early twentieth century that the link was definitively made, and not until the 1960s was the condition fetal alcohol syndrome (FAS) formally recognized (Jones, 1973).

Fetal alcohol syndrome and fetal alcohol spectrum disorders (FASD) refer to a range of conditions associated with prenatal alcohol exposure (PAE), leading to craniofacial abnormalities, growth problems, central nervous system dysfunction, and behavioral problems including deficits in learning, memory, attention span, communication, vision, and hearing in the developing fetus (CDC, 2015; Smith, 2014). Ethanol is capable of crossing the placenta, leading to a fetal alcohol blood content roughly two-thirds that of the mother. Due to the fetal liver's inability to detoxify ethanol, the fetus relies on the maternal hepatic metabolism to carry out this function. Ethanol will thus persist in the amniotic fluid for hours after the mother finishes consuming alcohol. Animal studies have shown harmful, irreversible defects as a result of blood alcohol levels as low as 0.02%, a level expected from an average adult drinking one unit of alcohol (Smith, 2014).

The medical community steadfastly notes that all FASD are preventable by simply abstaining from alcohol consumption, and it is the opinion of the World Health Organization and most Western governments that there is no safe time or amount for pregnant women to drink, especially given that there is an absolute 0% risk of developing FASD if the mother abstains from all alcohol consumption during pregnancy (CDC, 2015). Despite educational efforts to dissuade pregnant women from drinking and inform them of the risks to their child, prenatal alcohol exposure remains common. 7.6% of pregnant women reported drinking alcohol in the last 30 days, and 1.4% of pregnant women reported binge drinking in the same time period (CDC, 2015).

Estimates in the United States indicate 0.2 to 1.5 infants out of every 1,000 live births are born with fetal alcohol syndrome annually; rates of FAS amongst African-American populations of low socioeconomic background have been recorded as high 2.3 per 1,000 live births, and some American Indian reservations have seen incidence rates as high as 8.0 per 1,000 live births (CDC, 2015; Sampson, 1997; Abel, 1995). Fetal alcohol spectrum disorders (which encompass FAS and other, milder manifestations of teratogen exposure) affect 9.1 to 50 per 1,000 live births annually according to low estimates. High-risk populations, such as the Western Cape Province of South Africa, witness incidence rates as high as 68.0 to 89.2 per 1,000 live births (Sampson, 1997; May, 2007; Smith, 2014; CDC, 2015). Prenatal alcohol exposure leads to many more spontaneous abortions and stillbirths, especially when consumption occurs during the first trimester of pregnancy (CDC, 2015). The severity of FAS, FASD phenotype is integrally linked to the time at which ethanol exposure occurs. Research has shown that ethanol consumption

during the first two months of pregnancy – the first especially – leads to infants significantly more likely to be born below the 10th percentile for height, head circumference, both indicative findings of FAS, FASD (Day, 1989). This is consistent with findings implicating the neural crest in the pathogenesis of FAS, FASD: the process of neurulation occurs primarily late in the first month, early in the second month of pregnancy (Hall, 2009).

Both documentation of affected individuals and a wide array of animal models have suggested that prenatal alcohol exposure broadly suppresses neurodevelopment, including processes such as "neuronal induction, proliferation, survival, migration, synaptogenesis, and white matter formation" (Smith, 2014). While the degree and severity of symptoms is dependent on various genetic and environmental factors, the effect of ethanol is universally disadvantageous (May, 2011). Identifying the pathogenic mechanism of fetal alcohol syndrome spectrum disorders can be difficult; ethanol lacks a specific receptor, but rather interacts with hydrophilic portions of various proteins (Howard, 2011). The ability of ethanol to interfere with a large number of proteins is indicative of its broad-reaching and difficult to predict consequences on neurodevelopment. As a result, the quantity and frequency of ethanol consumption, the fetal stage of development during which prenatal alcohol exposure occurred, and other genetic and environmental factors determine the phenotype of fetal alcohol spectrum disorders (Smith, 2014).

The most archetypical FAS phenotype includes dual growth complications and neurodevelopmental deficits, coupled with a distinctive craniofacial appearance

(Sampson, 1997). The clearest neurodevelopmental deficits include decreased cranial size, especially the prosencephalon (which corresponds with a greater susceptibility of the cranial neural crest to ethanol than the trunk neural crest); given that there is a direct correlation between brain size and facial structure, the decreased volume of the prosencephalon is a likely contributor to craniofacial deformities, in addition to decreased migratory cranial neural crest cells (Sampson, 1997; Smith, 2014; Czarnobaj, 2014). Infants with FAS are generally below the tenth centile in terms of height and/or weight. While variability in the FAS phenotype is expected, there are three critical diagnostic criteria with regards to craniofacial abnormalities: a smooth philtrum, a thin vermilion, and small palpebral fissures. A myriad of other organs can also be affected, including the eyes and heart (Sampson, 1997; see Table 1, Figure 1).

Table 1. Typical symptoms in an individual with FAS or FASD. The FASD phenotype is highly variable, and it is unlikely that an individual with FASD will exhibit all, or even most, of the following (CDC, 2015; Riley, 2005).

Behavioral Deficits

Abnormal facial features	Difficulty with attention
Difficulty in school (particularly math)	Hyperactive behavior
Intellectual disability or low IQ	Learning disabilities
Low body weight	Poor coordination
Poor memory	Poor reasoning and judgment skills
Problems with the heart, kidneys, bones	Shorter than average height
Sleep and sucking problems as a baby	Small head size
Speech and language delays	Vision or hearing problems

Neurophysiological Deficits

Abnormal cellular migration	Agenesis of the commissure
Agenesis of the corpus callosum	Apoptosis of neuronal progenitors, neural crest cells
Basal ganglia anomalies	Brainstem anomalies
Decreased cerebral asymmetry	Decreased synaptogenesis
Decreased white matter formation	Lack of neuronal induction
Lack of neuronal proliferation	Microcephaly
Neuroglial heterotopias	Ventricular anomalies

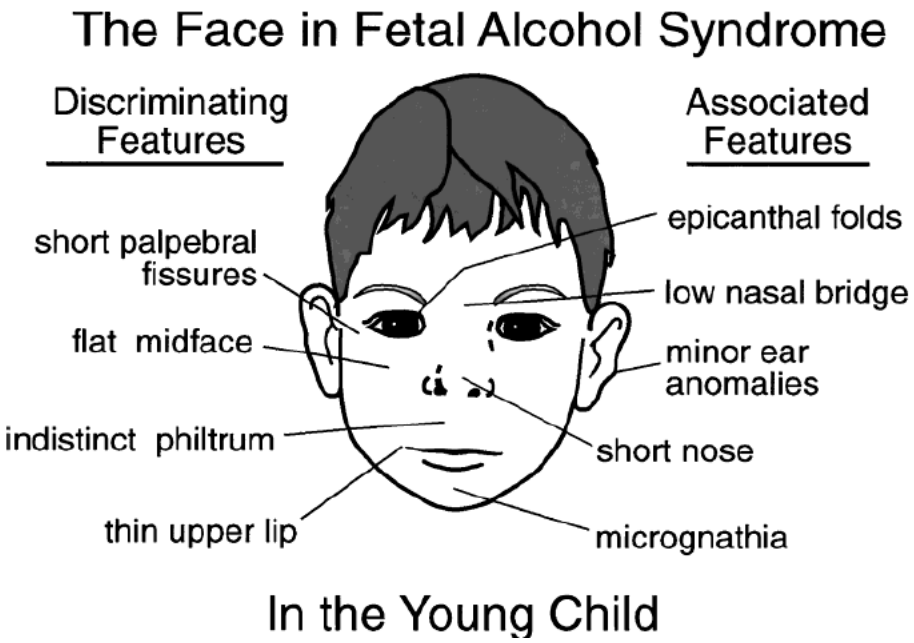

Figure 1. Characteristic craniofacial abnormalities of FAS, FASD (Sampson, 1997).

The Neural Crest

The neural crest refers to the folded structure of neural ectoderm, located at the articulation of the neural and epidermal ectoderm in the neurula-stage vertebrate embryo (Hall, 2009). Early in embryonic development, induction occurs in the ectoderm to produce three layers: the neural ectoderm (becoming the central nervous system), the non-neural ectoderm (becoming the epidermis), and the intermediate cells, the vast majority of which become the neural crest. Neurulation occurs due to signaling between the neuroectoderm, ectoderm, and paraxial mesoderm. During the process of neurulation, the border of the neural ectoderm folds upon itself, becoming the dorsal neural tube, and

the neural crest cells undergo an epithelial-to-mesenchymal shift, allowing them to migrate from the closing neural folds (Hall, 2009; Gammill, 2003).

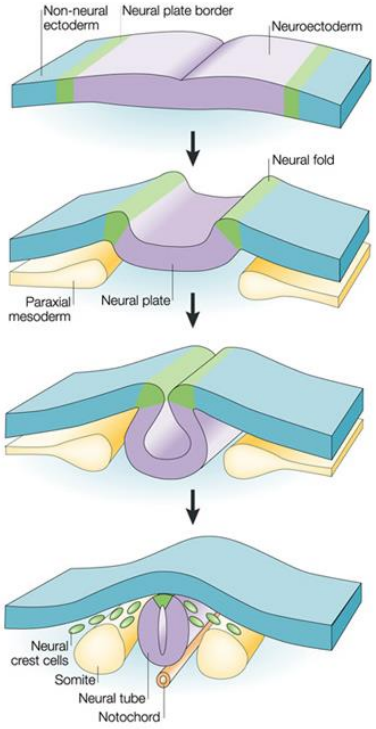

Figure 2. The developing embryo before, during, and after the process of neurulation. Neurulation is induced by the neural ectoderm (purple), non-neural ectoderm (blue), and paraxial mesoderm (yellow). The neural folds (green) elevate at the beginning of neurulation, leading to the neural plate closing in upon itself to form the neural tube. Delaminated neural crest cells (green) will migrate to their future destination (Gammill, 2003).

The neural crest is populated by neural crest cells (NCCs), a diverse array of cells that give rise to an inordinately large number of cell types. While some neural crest cells remain in the central nervous system where they originate, many of them migrate large distances away from the CNS, populating various tissues. These neural crest cells are migratory, multipotent cells, forming mature cells at the site of their emigration. The contribution of NCCs to a particular tissue or organ may be direct (providing cells and contributing to the mass of the organ), or indirect (providing an environment, often with an associated inductive signal, to a growing population of cells). Some researchers have

recommended that the neural crest be considered one of the germ layers (currently the ectoderm, mesoderm, endoderm, increasing their number to four), given the extraordinary number of tissue types originating from the neural crest (Hall, 2009; Gammill, 2003).

Table 2. A comprehensive, but not necessarily exhaustive, list of neural crest cell-derived tissues and cell types, either partially or in their entirety (Hall, 2009).

Cell Types

Adrenergic neurons	Angioblasts
Calcitonin-producing (C) cells	Cardiac mesenchyme
Cholinergic neurons	Chondroblasts, chondrocytes
Chromaffin cells	Fibroblasts (mesenchyme)
Glial cells	Melanocytes
Merkel cells	Mesenchymal cells
Odontoblasts	Osteoblasts, osteocytes
Parafollicular cells	Rohon-Beard cells
Satellite cells	Schwann cells
Sensory neurons	Smooth myoblasts
Striated myoblasts	

Tissues, Organs

Adipose tissue	Adrenal gland
Blood vessels	Brain
Cardiac septa, valves, aortic arches	Connective tissue*
Cornea	Craniofacial skeleton
Dorsal fin	Dentine
Endothelia	Eye
Heart	Parasympathetic nervous system
Peripheral nervous system	Smooth muscles
Spinal ganglia	Striated muscles
Sympathetic nervous system	Thyroid and parathyroid glands
Ultimobranchial body	

* of the thyroid, parathyroid, thymus, pituitary, and lacrimal glands

Objectives

An extraordinary amount of research has been performed over the last four decades, investigating the role of neural crest cells as the source of major pathologies of fetal alcohol syndrome and fetal alcohol spectrum disorders, and what the biochemical pathways perturbed by ethanol exposure are. This paper will help elucidate what the

prevailing models are, their implications, and future research that needs to be done. It will also discuss any potential treatment options, and the effect of low dose ethanol exposure both in animal models and human epidemiological studies, as the effect of moderate, social drinking on the fetus is a source of great debate.

RESULTS

The Role of G-Protein Coupled Receptors, Intracellular Calcium Release

The aforementioned facial features are neural crest derived, and animal models have generated evidence that apoptosis of premigratory and early migratory cranial neural crest cells occurs after ethanol exposure (Flentke, 2011; Ahlgren, 2002). Pre-migratory neural crest cells are the more susceptible to ethanol, with neural crest cells requiring larger doses after delamination and once migration has begun (Cartwright, 1998). Human studies and animal models show that there are different effects of ethanol exposure on neural crest cell populations depending on location. The cranial neural crest is most predominately affected, which makes sense given their role in populating the craniofacial complex and the abundant craniofacial abnormalities. Trunk neural crest cells have lesser changes, and no deficits are noted in melanocytes (Rovasio, 2002; Smith, 2014; Czarnobaj, 2014). Ultimately, while many factors control cephalic end-related abnormalities, the reduced number of cephalic neural crest cells due to apoptosis leads to a substantial proportion of FASD symptoms. This is confirmed by utilizing anti-apoptotic drugs, which rescue normal craniofacial features (Rovasio, 2002; Cartwright, 1998). The cell death is suggested to be apoptosis due to apoptotic marker labeling, pyknosis, and the ability to reverse apoptotic tendencies via treatment with caspase inhibitors (Cartwright, 1995; Cartwright, 1998; Flentke, 2014a). Programmed cell death is a natural part of embryogenesis. Research has indicated that ethanol-mediated apoptosis may be via inducing cell populations with a predisposition for apoptosis, hijacking a preexisting route for cell death (Sulik, 1988; Kotch, 1992).

In the cranial neural crest, ethanol has been shown to cause quick release of intracellular calcium stores within premigratory neural crest progenitors. This pathway seems to be mediated by a G-protein coupled receptor (GPCR) and associated downstream signaling, leading to activation of phospholipase Cβ and phosphoinositide-mediated release of calcium (Flenkte, 2011; Garic-Stankovic, 2005). The neural crest cell apoptosis is calcium-dependent; administration of calcium chelators before ethanol treatment is sufficient to prevent cell death. Inhibition of either $G_{\alpha i/o}$, phospholipase Cβ (PLCβ), or inositol triphosphate (IP_3) is also sufficient to prevent ethanol-induced apoptosis, supporting the theory that ethanol acts through this pathway. Activation of $G_{\alpha i/o}$ also causes release of $G_{\beta\gamma}$, which can itself directly activate the phosphoinositol-phospholipase C pathway (Debelak-Kragtorp, 2003; Garic-Stankovic, 2005). Release of intracellular calcium stores has been positively linked to neural crest cell apoptosis, however the target or targets for the increased calcium levels is still a source of some debate.

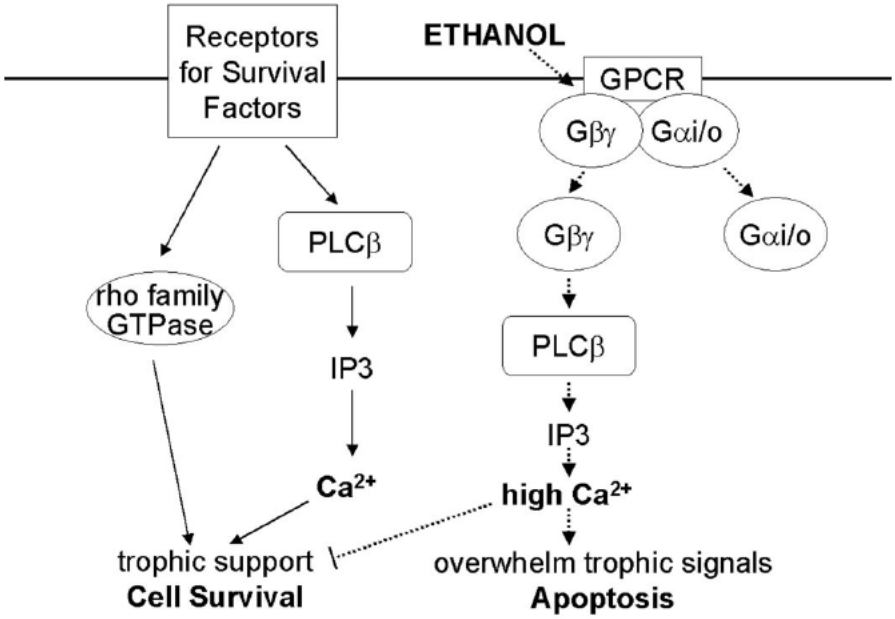

Figure 3. A proposed model of ethanol's actions through the phosphoinositol-phospholipase C pathway, as given by Garic-Stankovic (2005). Solid lines represent pathways of trophic support, while dotted lines indicate the actions of ethanol. It's believed that $G_{\alpha i/o}$ elevates IP_3 and Ca^{2+}, triggering apoptosis by saturating endogenous Ca^{2+} signaling systems.

Research has implicated CaMKII (Ca^{2+}/calmodulin-dependent kinase II). Neural crest cells showed quick, pervasive CaMKII activity after ethanol exposure, with activity increasing 300% above controls and occurring within 60 seconds of ethanol treatment. Ethanol's specificity for CaMKII was confirmed by utilizing antagonists for CaMKII, CaMKIV, and protein kinase C (PKC); only inhibitors for CaMKII prevented ethanol-induced apoptosis following ethanol exposure. Dominant-negative expression of CaMKII in these neural crest cells yielded similar results (Garic, 2011). Dominant-positive expression of CaMKII, meanwhile, caused apoptosis reminiscent of that seen after ethanol exposure. These results were seen in both chick and zebrafish embryos; this

indicates phylogenic conservation, and a similar mechanism may be present in humans as well (Garic, 2011; Flenkte, 2011).

A proposed model for the pro-apoptotic action of CaMKII is via the protein β-catenin, which has been shown to be phosphorylated and destabilized by CaMKII (Flentke, 2011; Smith, 2014). β-catenin is a component of the actin cytoskeleton, interacting with E-cadherin and promoting cell adhesion; more importantly, it also acts as a mediator of transcription, interacting with transcription factors TCF and LEF, ultimately providing trophic support for cells (Flentke, 2011; MacDonald, 2009). Ethanol-mediated decrease in β-catenin causes decreased expression of *Slug*, *FoxD3*, and *Wnt6*, factors necessary for neural crest survival and progression. Loss of these pathways causes pervasive apoptosis of the cranial neural crest and associated craniofacial abnormalities. Blocking the release of intracellular calcium or exogenously expressing β-catenin in ethanol-exposed cells rescues the neural crest cells from apoptosis, indicating the source of NCC losses. (Flentke, 2011). These results are consistent with genetic models for β-catenin deficiency, which show similar cranial neural crest death and phenotypic craniofacial abnormalities (Brault, 2001). Researchers discovered three highly conserved CaMKII target sites on β-catenin, indicating that CaMKII phosphorylates β-catenin directly, causing its degradation and subsequent apoptosis. Inhibiting other effectors that destabilize β-catenin, such as calpain, glycogen synthase kinase-3β, and protein kinase C were insufficient to ameliorate neural crest cell death, indicating that CaMKII's direct phosphorylation of β-catenin is sufficient to mediate cellular apoptosis (Flentke, 2014a). These results have been confirmed in both chick and zebrafish

embryos, indicating an evolutionarily-conserved mechanism (Flentke, 2014b). These results also help justify results indicating that the cranial neural crest is more susceptible, and that earlier neural crest cell populations are sensitive due to preexisting apoptotic pathways, which coincides with research indicating that drinking during the first two months of pregnancy – when normal, endogenous apoptosis of neural crest cells would occur – leads to poorer outcomes (Abel, 1995; Smith, 2014; Hall, 2009). Wnt signaling is part of endogenous cell death, and increased Ca^{2+}/abnormal Wnt signaling due to ethanol acts synergistically with these pathways. Yet, the downstream mechanism by which Wnt mediates apoptosis remains a mystery (Smith, 2014).

Migration Abnormalities

Neural crest cells have been shown to experience difficulty migrating following exposure to ethanol. *In situ* hybridization studies on *Xenopus* explored neural crest cells expressing *Twist* and *Slug*, two genes linked to neural crest migration. In control embryos, these cells are found to migrate ventrally, but were dorsally located following prolonged ethanol exposure. These results indicate disruption in normal neural crest migration. These effects were dose-dependent, with higher ethanol concentrations leading to clusters of cells located increasingly more dorsally (Shi, 2014). Migratory anomalies have been noted amongst neural crest cells at all neuraxis levels (Rovasio, 1995; Rovasio, 2002). Ethanol exposure also causes neural crest cells to migrate in lesser quantities from the prosencephalon, mesencephalon, and rhomboencephalon (Cartwright, 1995; Rovasio, 2002). *In vitro* and *in vivo* experiments have documented that ethanol treatment leads to

translocation of the neural crest cells, which did migrate properly, towards the lumen of the neural tube; this abnormal migratory pattern differs from standard movement, which is towards the lateral sides of the neural tube (Rovasio, 1995). Experiments in *Xenopus* showed that ethanol treatment causes decreased numbers of trunk and especially cranial neural crest cells migrating (Czarnobaj, 2014). Despite the effects on migration, ethanol does not seem to have a significant effect on neural crest cell induction, an early embryonic process (Shi, 2014).

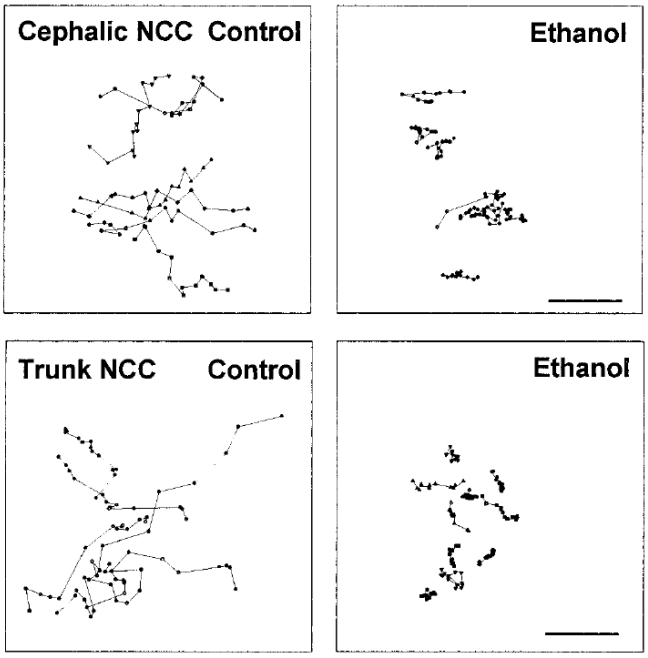

Figure 4. Real-time diagram of migratory tracts of cephalic, trunk neural crest cells in culture. Figure is given in 6 hour increments (indicated by dots), as either controls or after treatment with 150 mM ethanol. Neural tube explants are located on the left side and migrate towards the right; cephalic cells are over 24 hours, trunk cells over 48 hours. There is a visually apparent decrease in motility in ethanol treated cells compared to controls. Bars = 100 µm (Rovasio, 2002).

Cells of the neural crest are interconnected by zonula adherens, which occurs when a cell-cell interface is joined via connections to the intracellular actin cytoskeleton. By the "fixed cortex" model of mesenchymal cell migration, cells have protruding apical

processes, extending into the lumen. The cell will eventually follow the processes, sliding past the zonula adherens, and the trailing end of the cell will become detached. Ultimately, blocking interaction of neural crest cells with the basal extracellular matrix causes a transformation of the apical neural crest cell into the leading end; this causes movement towards the lumen of the neural tube (Bilozur, 1989; Rovasio, 2002). These perturbations between the extracellular matrix and neural crest cells resembled those observed after ethanol administration.

Research by Czarnobaj (2014) investigated the effects of low-dose ethanol on *Xenopus* cranial and trunk neural crest cells and the subsequent effects on cell motility and migration, with the conclusion that ethanol, even at low doses, perturbs both cranial and trunk neural crest cells, although cranial NCCs to a greater extent. They also noted a difference in how cranial and trunk neural crest cells respond to ethanol when on collagen and fibronectin stratums. Yet, it remains unclear whether or not ethanol disrupts the extracellular matrix environment, or whether the effects on migration are mediated entirely by the cells (Czarnobaj, 2014). Interesting, too, is that the migratory effects have been repeatedly shown not to be transient; irregular migratory patterns persisted for 24 hours beyond ethanol removal (Czarnobaj, 2014; Rovasio, 2002).

The actin cytoskeleton of neural crest cells "undergoes disruption, thickening, collapse, and fragmentation" (Oyedele, 2013) following ethanol exposure, correlating to the aforementioned migratory abnormalities. Actin filaments comprise a cross-linked structure, which lines the cytoplasmic face of the plasma membrane, as well as fibers that run through the cytosol and into lamellapodia. Ethanol (in epithelial cells) has been

shown to cause collapse of the actin cytoskeleton, with an inverse relationship between ethanol concentration and polymerized cytoskeletal actin. The prevailing theory is that ethanol disrupts polymerization into F-actin, necessary for focal adhesions and migration (Banan, 2000). Further research suggested that ethanol's disruptive role on cytoskeletal actin is not only dose-dependent, but also embryonic stage dependent. Indeed, experiments in chick embryos showed that the teratogenic effect of ethanol on cytoskeletal actin was observed only in embryonic stage 9, not stages 8 or 10. As the researchers noted, "the fact that similar concentrations of ethanol affect different stages of embryos differently, or conversely that embryonic or fetal cells at the same stage of development respond differently to lower versus higher ethanol concentrations, underlies the complexity of ethanol's teratogenic effects" (Oyedele, 2013).

The effects of depolymerization and cytoskeletal remodeling include retraction of filopodia and decreased numbers of focal adhesions, with actin bundles expressing less arborization and greater thickness (Banan, 2000; Oyedele, 2013). Intracellular calcium is also a mediator of cytoskeletal reorganization, and the ethanol-induced increases in intracellular calcium described before may play a role in neural crest cell migratory difficulties following ethanol exposure (Smith, 2014).

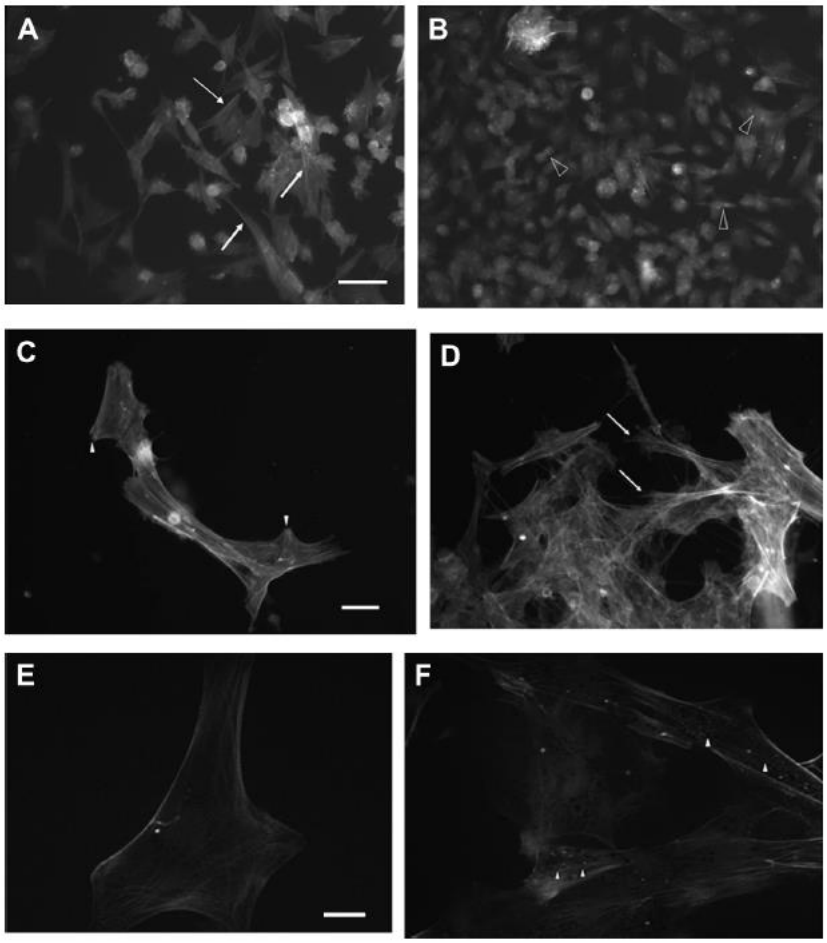

Figure 5. Avian cranial neural crest cells, rhodamine-immunolabeled. (A), (C), and (E) are untreated controls; (B) is 0.4% v/v ethanol-treated cells, in culture 48 hours; (D) is 0.2% v/v ethanol-treated cells, in culture 96 hours; (F), 0.2% v/v ethanol-treated cells, in culture 6 days. Arrows in (A) indicate actin filaments, which are not present in (B); arrows in (B) indicate cells in late mitosis. (D) shows that after 96 hours in ethanol-treated culture, focal adhesions are lost (indicated by arrows) and ethanol-treated cells begin to congregate. Arrows in (F) indicate areas of cytoskeletal disorganization and breakdown. Scale bars: (A), (B): 5 µm; (C), (D): 3 µm; (E), (F): 2 µm (Oyedele, 2013).

Cell adhesion molecules (CAMs) have been implicated in the ability of neural crest (and other) cells to migrate, as they create spatial organizations necessary for proper motility. Cell adhesion molecules are found on the extracellular surface of the plasma membrane and, as the name suggests, play an integral role in tissue structure by interconnecting cells with each other and the extracellular matrix. Research has

implicated L1-CAM as a target for ethanol, and ethanol concentrations as low as 1 mM have created improper cellular adhesion (Ramanathan, 1996). Human L1-CAM is part of the immunoglobulin superfamily, and has roles in neurite outgrowth and formation of bundles; it's heavily expressed in growing cones of axons, and aids in migration (Wong, 1995).

Experiments utilizing recombinant human cDNA showed that L1-transfected mouse fibroblasts showed increased cell-cell adhesion, an improvement ablated by ethanol treatment. (Ramanathan, 1996; Rovasio, 2002). These interactions were seen in ethanol, as well as n-propanol and n-butanol, but not n-alcohols of 5 or more carbons, indicating that ethanol (and shorter n-alcohols) interact with a small, hydrophobic portion of the L1-CAM protein (Ramanathan, 1996). Mental retardation, hydrocephalus, and absence of the corticospinal tract and corpus callosum are both defining characteristics of severe FAS and genetic mutations in human L1-CAM. The congruence of phenotype and dysfunction of L1-CAM suggests that a potential role for L1-CAM in the pathogenesis of FAS (Wong, 1995; Ramanthan, 1996; Rovasio, 2002).

The role of retinol as a teratogen and as the mediator of ethanol-induced disruption of neural crest cell migration will be discussed later.

Folic Acid and FASD

Dysregulation of folic acid has been known – independent on any ethanol involvement – to be a strong mediator of neurocristopathies and severe birth defects (Etchevers, 2006). Research has linked folic acid metabolism to the teratogenesis of

ethanol, with ethanol-treated mouse embryos expressing abnormal microRNAs (miRNA) and downregulated Hoxa1 expression; these defects were ameliorated by co-incubation with folic acid (Wang, 2009). Similar studies indicate that folic acid supplementation reduces ethanol-associated microcephaly; this may be mediated by folic acid reversing the downregulation of proteins of various metabolic processes, including isocitrate dehydrogenase (of the tricarboxylic acid cycle), serine/threonine protein phosphatase (involved in signal transduction), and proteins of the proteasome (Xu, 2008). The means by which folic acid rescues a wild-type phenotype is unclear. Early stages of embryogenesis require maximal nucleic acid and protein synthesis. Folic acid is necessary for these processes as a cofactor, and thus may reverse ethanol's effects by either directly upregulating protein expression, or may upregulate other proteins to better handle the ethanol-mediated oxidative stress (Xu, 2008). Ethanol also increases renal excretion of folic acid by blocking its reabsorption, and supplementation with additional folic acid may help undo these effects (Muldoon, 1994).

In addition to affecting folic acid metabolism, ethanol has been shown to produce hyperhomocysteinemia in the *Xenopus* fetus during the migration stage of neural crest cells (Stickel, 2000; Shi, 2014). Elevated homocysteine levels disturbs neural crest cell migratory behavior, possibly through a homocysteine-sensitive, NMDA-like receptor expressed by early migratory neural crest cells; hyperhomocysteinemia leads to defects of the neural tube and craniofacial abnormalities (Brauer, 2002). Supplementation of ethanol-exposed *Xenopus* fetus with 5-methyltetrahydrofolate, the most biologically active form of folic acid, reduces homocysteine levels and rescues migratory behavior of

the neural crest cells (Shi, 2014). Folic acid seems to rescue neural crest cell migratory abilities via its direct actions on various proteins and nucleic acids, and by reducing homocysteine levels, which are by themselves teratogenic; the relative contribution of each of these pathways to the dysfunction of neural crest migration remains unclear. However, the abundance of research does indicate that mothers who have consumed ethanol during pregnancy would be well-advised to supplement with folic acid, if they are not doing so already (Shi, 2014).

Trophic Support

Defects to the prosencephalon are severe enough to categorize FAS along the holoprosencephaly (HPE) spectrum in many cases (Sulik, 1984). Holoprosencephaly is a failure of the fetal prosencephalon to bifurcate into the two cerebral hemispheres. Premature alcohol exposure, neural crest cells, and expression of *Shh* seem to be correlated, specifically on the midline specification of cerebral, craniofacial, and oral structures (Aoto, 2008). Animal models have shown that *Shh* expression in the gastrula organizer node progresses to the axial mesendoderm, which gives rise to the notochordal plate (Echelard, 1993). Specifically, prechordal plate (PrCP) cells, which comprise the anterior-streak derived axial mesendoderm, migrate to below the anterior neural plate, and are pivotal for proper development of the brain and facial structures (Aoto, 2009).

Various mutations have been implicated in the pathogenesis of HPE, perhaps most significantly *Sonic Hedgehog* (*SHH*) (Higashiyama, 2007). Prenatal alcohol exposure decreases *Shh* expression in these structures (Echelard, 1993). Despite this

evidence, the molecular pathway and role of *Shh* remains unclear, as while decreased levels of *Shh* were reported in chick (Ahlgren, 2002), mouse (Higashiyama, 2007), and zebrafish (Li, 2007) embryos after treatment with ethanol, *Xenopus* reported higher levels of *Shh* (Yelin, 2007). However, current theories suggest that if ethanol is consumed during a specific developmental window for the prechordal plate, it will result in the HPE phenotype (Higashiyama, 2007). The current model suggests that *Shh* is necessary for PrCP cell survival; a lack of PrCP cells, which are critical for the process of neurulation, would disrupt the neurodevelopment and produce the HPE phenotype. Thus, early prenatal alcohol exposure may lead to craniofacial abnormalities indirectly, via disruption of the neuroectoderm rather than the neural crest (Aoto, 2008; Aoto, 2009; Hall, 2009; Smith, 2014).

However, *Shh* has been consistently shown to prevent apoptosis in neural crest cells and other populations (Smith, 2014). *Shh* is most prevalently expressed in the cranial neural crest, and embryos treated with ethanol or anti-*Shh* antibodies exhibit similar phenotypes: decreased head size, craniofacial abnormalities, and apoptosis of cranial neural crest. Exogenous *Shh* also rescued ethanol-treated neural crest cells from death (Ahlgren, 2002; Aoto, 2008). The facial skeletogenic neural crest (FSNC) is the rostral portion of the neural crest that gives rise to skeletal structures of the face, such as the maxilla and mandible. Removal of the FSNC or the preceding cranial neural crest leads to a phenotype strongly resembling HPE (Brito, 2006). Inhibition of *Shh* expression leads to destruction of the FSNC, as well as associated neural crest cells; researchers have concluded *Shh* is vital for FSNC cell survival during migration to the branchial arch

(BA1). This Shh appears to be derived from the anterior ventral foregut endoderm (Brito, 2006).

However, researchers noted that lack of *Shh* signaling was not sufficient enough to produce the ethanol phenotype: in severe ethanol-induced HPE, there is near-complete ablation of the telencephalon and eyes, which is not observed in *Shh*$^{-/-}$ embryos. This is consistent with results showing that, in mice models, ethanol-induced decreases in *Shh* expression were exhibited only in the anterior prechordal mesendoderm, triggering apoptosis (Aoto, 2008).

Post-translational modification of fetal proteins is critical for the sequential order of embryogenic steps, and sterol and fatty acid-modified *Shh* confers upon it different functionality, which is vital for its proper formation, translocation, and function, depending on its fetal location and timing (Li, 2007). Research has indicated that these post-translational modifications may be the target of ethanol as a teratogen in the *Shh* pathway. This is exemplified by research showing that individuals with Smith-Lemli-Opitz syndrome (SLOS), an autosomal recessive condition resulting in 7-dehydrocholesterol reductase deficiency and dysfunctional cholesterol synthesis, show a characteristic phenotype similar to that of FAS-associated HPE (Kelley, 1996). Similar results are seen by exposing embryos to agents that block cholesterol synthesis or selectively decrease its levels (Cooper, 2003). The prevailing theory is that ethanol acts by disrupting HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase activity, causing decreased free cholesterol in fetal cells and circulation, as well as by inhibiting hepatic ACAT (acetyl-coenzyme A acetyltransferase); both HMG-CoA reductase and

ACAT are instrumental in proper cholesterol biosynthesis, and their inhibition could result in defective *Shh* leading to the HPE phenotype seen in FAS (Polo, 2003). Other research has definitively shown that ethanol, even in low doses, inhibits covalent modification of *Shh* by cholesterol, disrupting Hedgehog (Hh) signal transduction and causing a constellation of symptoms mirroring FASD; supplementation with cholesterol rescues embryos from this fate (Li, 2007).

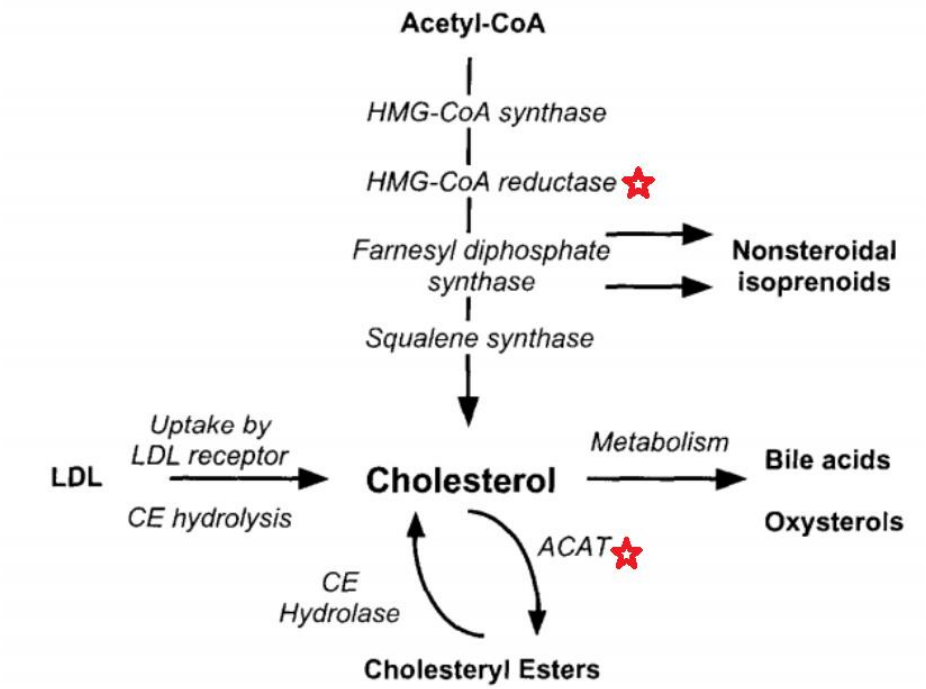

Figure 6. In mammals, the metabolic, transport pathways controlling cholesterol metabolism. Free cholesterol is either synthesized *de novo* from acetyl-CoA, or can be obtained by hydrolyzing cholesterol esters present in the cell (often via LDL molecules). Ethanol is believed to disrupt both HMG-CoA reductase and ACAT (both marked by a red star), and the subsequent cholesterol dysfunction contributes to the pathologies of FAS, FASD.

Contradicting the role of post-sterol modification as the dysregulation of *Shh* are results indicating that, while ethanol does disrupt *Shh* signaling leading to neural crest cell apoptosis at some embryonic stages, it is not a universal occurrence. *Shh*-deficient chick neural crest cells at the onset of migration will exhibit apoptotic tendencies, a

behavior ameliorated by exogenous *Shh* administration. However, similar studies on mouse embryos at the same stage found that ethanol did not perturb *Shh* levels, and yet neural crest cell death and craniofacial defects still occurred (Yamada, 2005). Another study found that rat cranial neural crest cells, the population responsible for craniofacial abnormalities, actually express increased *Shh* following ethanol exposure (Wentzel, 2009). One would expect the proposed model (HMG-CoA reductase and ACAT interference) to apply more broadly, throwing its validity into question. Additional research needs to be performed to gauge the role of *Shh* and post-sterol signaling in the FASD phenotype.

Further confounding these results, however, are other studies indicating that ethanol-mediated decreases in *Shh* are via protein kinase A (PKA) activation. PKA is an endogenous inhibitor of *Shh* signaling in the neural tube. In anterior PME cells of mice, ethanol has been shown to activate adenylyl cyclase activity, thereby upregulating the levels of cyclic AMP (cAMP), which in turn activates PKA. Ethanol also causes translocation of PKA to the nucleus, where it phosphorylates transcription factor CREB, which causes a subsequent drop in *Shh* levels; these results are reversed with treatment of a PKA inhibitor, indicating that PKA is indeed the pathway by which ethanol causes *Shh*-induced HPE (Aoto, 2008). However, the same researchers investigated the role of oxidative stress in the development of ethanol-induced HPE. Vitamins C and E (L-ascorbic acid and DL-α-tocopherol, respectively) are dietary antioxidants, and supplementation of mice with these compounds in conjunction with ethanol exposure reduced the incidence of HPE from 25% to 5.2% (Aoto, 2008). How (or if) the three

proposed models – post-translational sterol modification, PKA signaling, and oxidative stress – act together to produce HPE is unclear.

As discussed previously, the cranial neural crest cells seem to be particularly susceptible to the teratogenic effects of ethanol, with the trunk neural crest affected to a lesser degree and other neural crest cell populations not at all. Investigations into the genetic expression in the cranial versus trunk neural crest cell provide some insight into the fundamental differences between these cell populations. In the cranial neural crest of rats, ethanol administration increases expression of Shh, Bmp-4, and T box-2; these effects are not seen in trunk neural crest (Wentzel, 2009). Bmp-4 is of particular interest, as it promotes embryonic apoptosis (Graham, 1996) and modulates L1-CAM expression (Wilkemeyer, 1999), both of which have been independently identified as contributing to the FASD pathology. Wentzel (2009) also found that cranial neural crest cell Hox genes (Hoxa1, Hoxa4, Hoxa5, but not Hoxa2) were downregulated (almost no expression), while the trunk neural crest cells had upregulated expression of all 4 Hox genes (Wentzel, 2009). These effects begin to elucidate the particular sensitivity of the cranial neural crest to teratogen exposure, but not other regions.

Various other trophic factors have been shown to function in neural crest proliferation, migration, and survival. Experiments using chick embryos investigated the developmental window between the beginning of migration through the closure of the neural tube, during which the cranial neural crest is especially susceptible to ethanol exposure. Researchers discovered that ethanol-mediated apoptosis of the neural crest during this developmental window was reversed by treatment with neurotrophin-3 (NT3),

which aligns well with the model proposed by Garic-Stankovic (2005) regarding the role of GPCRs and intracellular calcium (Jaurena, 2011). NT3 binds to its receptor, tropomyosin receptor kinase C (TrkC), which is a receptor tyrosine kinase. Similar restorative results were seen in mouse embryos by co-incubating with ethanol and HB-EGF, which binds to ErbB1, ErbB4 – both part of the epidermal growth factor (EGF) receptor family – which are also both receptor tyrosine kinases (Kilburn, 2006). GM1 ganglioside also has protective effects, and will be discussed later (Chen, 1996b).

The Role of Retinol, Retinoic Acid

Retinoic acid is synthesized from retinol (vitamin A), and regulates many steps of embryonic development (Yelin, 2004). Within the central nervous system, retinoic acid is considered vital for "stimulation of axon outgrowth in particular neuronal sub-types, the migration of the neural crest, and the specification of the rostrocaudal position in the developing CNS (forebrain, midbrain, hindbrain, spinal cord)." Lack of vitamin A or retinoic acid leads to catastrophic birth defects (Maden, 1992; Madden, 1996). Retinoic acid has been shown to be vital in gastrulation, as well as in establishing neural axes during CNS development, primarily through modulation of the *Hox* genes. Retinoic acid administration has been shown to induce *Hox* expression, which is vital in determining the axes of the neural crest and other embryonic structures (Maden, 1996; Hall, 2009). In experiments utilizing quail embryos, neural crest cells were produced normally in the absence of retinol, but following migration, apoptotic tendencies were exhibited. Retinoic acid is thus believed to preserve and maintain crest-derived neuronal precursor cells.

Failure of this preservation leads to malformations in the dorsal root ganglia and the neural tube, which becomes unable to extend neurites into the periphery (Maden, 1996). Mammalian models have shown ethanol to be a competitive inhibitor for retinol dehydrogenase, which normally converts retinol to retinoic acid. If ethanol and retinol are both substrates for the retinol dehydrogenase enzyme, then prenatal alcohol exposure could lead to temporary decreases in retinoic acid while the enzyme preferentially detoxifies ethanol to acetic acid (Duester, 1991).

These effects are coupled to the fact that ethanol treatment increases levels of retinol by inhibiting retinol dehydrogenase's ability to detoxify it (Duester, 1991). Somewhat paradoxically, retinol itself acts as a teratogen. Embryonic treatment of retinol leads to craniofacial abnormalities, including a cleft palate; this is believed to be due to faulty neural crest cell migration. These suspicions are supported by animal model studies utilizing quail embryos: neural crest cells exposed to hypervitaminosis A gravely affects their locomotory abilities. As a whole, retinol-exposed cells exhibit significantly less mobility, with lamellipodia unable to remain extended. This effect is coupled to, and in part due to, a culmination of factors, including reduced cellular outgrowth, dysfunctional or sometimes absent lamellipodia, lack of cellular adhesion to the extracellular matrix, and dysmorphologies in shape, deviating from a normal triangular shape to fusiform. (Thorogood, 1982).

In amphibians, the Spemann's organizer is a gastrula-stage structure that is capable of producing a secondary body axis if transplanted; it induces dorsalization of ventral mesoderm and specifies the neuroectoderm. In *Xenopus*, the Spemann's organizer

utilizes retinoic acid signaling during the gastrula stages, and as a result this structure may be susceptible to ethanol exposure during this embryonic period. Thus, some of the embryonic abnormalities attributed to prenatal alcohol exposure may be the result of dysfunctional retinoic acid signaling (Yelin, 2005). Disorder of Spemann's organizer contributes to malformation of the rostro-caudal and dorsal-ventral axes, and increased size of the prechordal plate and notochord coupled with decreased spinal cord size (Yelin, 2005; Yelin, 2007).

Oxidative Stress

Ethanol has been repeatedly shown to have adverse effects on glucose metabolism in the body. Ethanol consumption has been shown to reduce levels of necessary proteins, such as the transport protein Glut1, perturb the mitochondria and reduce the enzymes of the electron transport chain, and reduce available ATP stores, thereby leading to oxidative stress (Cunningham, 2003).

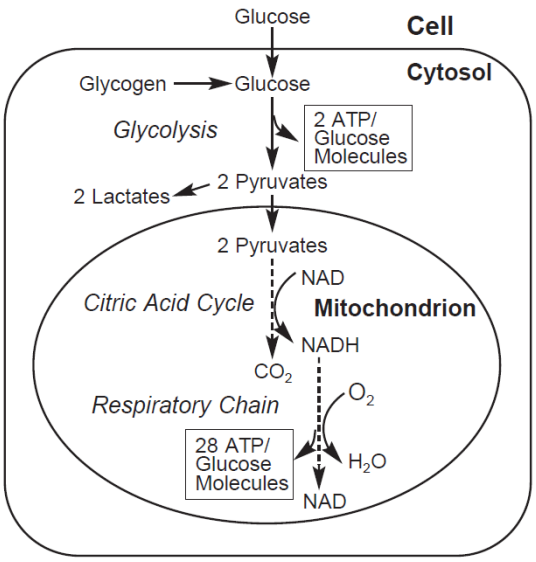

Figure 7. Aerobic, anaerobic cellular respiration in humans. Glycolysis is a metabolic process conducted in the cytosol, in which glucose is catabolized into two molecules of pyruvate, generating two molecules of ATP; the pyruvate then, in the presence of oxygen, enters the mitochondria and is catabolized further by the tricarboxylic acid cycle to produce NADH, which is fed into the electron transport chain to generate the majority of ATP (Cunningham, 2003).

Oxidative stress can generate reaction oxygen species (ROS), which are cytotoxic. Many cells counteract reactive oxygen species through the actions of the enzyme superoxide dismutase, which converts superoxide (O_2^-) into hydrogen peroxide (H_2O_2), a less dangerous species (which is further degraded by other enzymes). Research has shown that ethanol treatment on the neural crest generates significant levels of reactive oxygen species (Davis, 1990; Chen, 1996a; Kotch, 1995). Immunoassays for superoxide dismutase in neural crest cells indicate a lack of this enzyme, which may explain the neural crest's susceptibility to a host of teratogens, including ethanol (Davis, 1990). Reactive oxygen species production led to craniofacial abnormalities and neural crest cell death reminiscent of ethanol treatment. The teratogenic effects of ethanol on the neural crest were ameliorated by supplementation with exogenous superoxide dismutase and

other free radical scavengers, such as α-tocopherol and catalase (Davis, 1990; Kotch, 1995; Chen, 1996a). A phenotype similar to that of ethanol administration on neural crest cells was achieved after treatment with xanthine/xanthine oxidase, a system that produces free radicals. These effects were ameliorated by superoxide dismutase and catalase (free radical scavengers). Rescuing the neural crest cells from death suggests that "free radicals play a significant role in ethanol-induced NCC death" (Chen, 1996a).

This generation of free radicals after ethanol administration produces loss of microvilli, leakage of radiolabeled chromium (Cr), and other pathologies associated with the cell membrane (Davis, 1990). The notion that the cell membrane is a major target of ethanol-mediated toxicity is supported by increased fluidity of the membrane following ethanol exposure. This effect is reversed by exogenous gangliosides, glycolipids whose function is to stabilize plasma membranes (Chen, 1996b). There may also be some cross-talk between the production of free radicals and apoptotic tendencies: N-acetylcysteine (NAC) is an antioxidant agent, and has been shown to decrease ethanol-derived apoptosis in the neural crest (Wentzel, 2009).

Why exactly the neural crest either generates more reactive oxygen species or is particularly susceptible to their pathogenic actions is unclear (Smith, 2014). Some researchers have investigated the role of free iron in this pathway. Generally, free iron does not exist in the body; it's bound to functional proteins such as hemoglobin, transport proteins such as transferrin, or storage proteins such as ferritin. However, ethanol is oxidized by alcohol dehydrogenase into acetaldehyde, producing NADH; NADH liberates stored iron from ferritin, increasing free iron levels in the cell. Ethanol also

prevents conversion of reactive ferrous iron (Fe^{2+}) into non-reactive ferric iron (Fe^{3+}). Additionally, the production of acetaldehyde and its subsequent catabolism often generates free radicals, which exacerbates this process (Shaw, 1998; Chen, 2000; Shaw, 1999). Cellular free iron can produce free radicals via the Fenton reaction, seen in Figure 5. Lipid peroxidation was found after release of free iron from ferritin; this process was confirmed to be iron-dependent, as the effects were reversed by adding the iron chelator deferoxamine (Double, 1998). These results are consistent with research on ethanol's effects on production of reactive oxygen species and their subsequent effects on the cell membrane.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH$$

Figure 8. Free iron (Fe^{2+}) can generate free radicals via the Fenton reaction.

These results are especially relevant given that chronic ethanol consumption increases iron absorption from the intestines and iron stores in the liver; fetal iron also increases following ethanol exposure (Chen, 2000). While these results suggest a potential link, they have not definitively connected the generation of free radicals to dysfunctional iron metabolism, and more research is required to clarify the relationship.

Genetics

FAS and FASD are highly variable diseases, in their symptoms, degree of severity, and in how alcohol causes their presentation. Anecdotal evidence indicates that some women who binge drink throughout pregnancy have children who present with few

if any symptoms along the FASD spectrum, while some women who ingest relatively low amounts of alcohol present with severe FAS or stillbirth. There is strong evidence that the etiology of FAS and FASD is complicated, with evidence that the drinking patterns and peak concentration, maternal age, hormonal levels, and nutrition, and genetics are all significant co-factors (Smith, 2014; Debelak, 2000).

Studies on twins of alcoholic mothers showed that, despite identical alcohol exposure in utero, monozygotic twins showed significantly more concordance with regards to FASD phenotype than dizyogotic twins (Streissguth, 1993). Additionally, in chicks (Debelak, 2000) and zebrafish (Loucks, 2004), the extent of craniofacial abnormalities following ethanol exposure is highly variable, and in zebrafish has been linked to specific genetic variables. Most of these are involved in the metabolism of alcohol – alcohol dehydrogenase, the first (and rate-limiting) step in detoxifying ethanol, is perhaps the most widely implicated – but investigators are likely to find many more in subsequent studies. This indicates a strong genetic component that is likely to persist in humans, and explains why some individuals with FASD also have craniofacial defects (Smith, 2014; Debelak, 2000).

Platelet-derived growth factor (Pdgf) is one of many signaling molecules that modulates neural crest cell migration, survival, and ultimately the development of the craniofacial mesenchyme. It is a receptor tyrosine kinase, activating PI3K and mTOR to mediate its downstream effects. Research showed that the pdgfra heterozygotes and mutants show neural crest cell migratory abnormalities resulting in cleft palate, and in the presence of ethanol, even more severe craniofacial anomalies. The researchers propose

that wild-type pdgf serves a protective function for neural crest cells that is deficient in those with the pdgfra allele. Heterozygotes and pdgfra mutants are even more susceptible to the teratogenic effects of ethanol than wild-type individuals (McCarthy, 2013). Studies on a South African population have shown that presence of the alcohol dehydrogenase B*2 allele, which has greater enzymatic activity and thus lessens ethanol's half-life, results in a lesser incidence of FAS and FASD, further supporting the notion that the pathogenesis following prenatal ethanol exposure is both environmental and genetic (Viljoen, 2001).

DISCUSSION

The discovery of the exact mechanisms involving ethanol's effect as a teratogen are of paramount importance; as many as 1.5 and 50 live births per 1,000 are affected by fetal alcohol syndrome and fetal alcohol spectrum disorders, respectively. The total annual cost on both the family and society due to FASD was $14,342 per child annually, translating to as much as $230 billion in the United States (Sampson, 1997; CDC, 2015; Popova, 2012). The effect of ethanol on neural crest cells has been explored vigorously, with more than a half dozen models describing its effects. Neural crest cells are implicated most directly with the craniofacial abnormalities seen in individuals with FAS, and some with FASD, and likely contribute to a host of other pathologies of FASD, including visual and auditory, cardiac, and neurological deficits (Hall, 2009).

Substantive research has shown that the cranial neural crest is more susceptible to ethanol exposure (Rovasio, 2002), inducing apoptosis (Cartwright, 1995; Cartwright, 1998). Current models suggest that ethanol activates a GPCR, leading to activation of phospholipase Cβ and subsequent increase in intracellular calcium levels (Flentke, 2011; Garic-Stankovic, 2005). Research has implicated CaMKII as the source of calcium-mediated apoptosis, possibly by its phosphorylation of β-catenin and subsequent modulation of transcription of trophic factors (see figure 9). However, Flentke (2011) notes that GSK3β is an inhibitor of β-catenin activity as well, and while it has been implicated in FASD, it is considered calcium-independent. How GSK3β, as well as other potential β-catenin modulators such as PKC and calpain, affect β-catenin activity and contribute to the FASD phenotype is unclear. It remains unknown, as well, what (if any)

activity $G_{\alpha i/o}$ has, and which GPCR is affected by ethanol, perhaps the most salient mystery of all. While a near-complete picture has been painted with regards to the GPCR pathway of ethanol neural crest cell toxicity, more research is necessary to fill in the canvass.

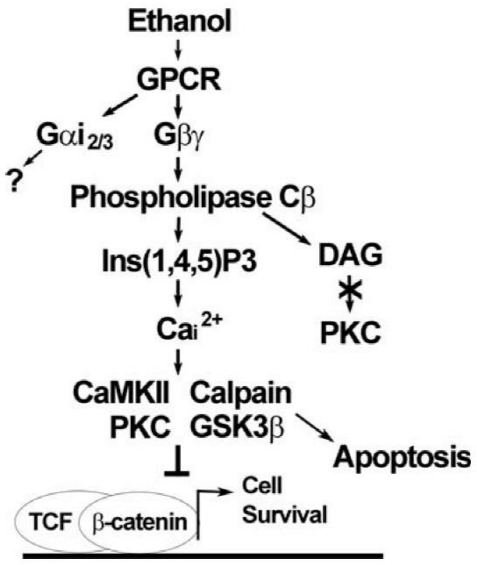

Figure 9. Proposed model by which ethanol induces neural crest cell apoptosis via a GPCR. Ethanol stimulation causes a rapid (within seconds) increase in intracellular calcium levels, believed to be caused by a phospholipase C-mediated pathway. CaMKII is known to cause effects on β-catenin, a transcription factor regulator, but other mediators may have a role as well. Both the GPCR, and all the relevant parties regulating apoptotic behavior, are unknown (Flentke, 2011).

Migratory difficulties of neural crest cells are well known, occurring at all neuraxis levels (Rovasio, 1995; Rovasio, 2002). The prevailing theory is that disturbances in the migration of neural crest cells is due to dysfunction of the actin cytoskeleton; F-actin polymerization is decreased in the presence of ethanol (Banan, 2000; Oyedele, 2013). However, the mechanism by which ethanol perturbs F-actin polymerization is unclear, and is a necessary avenue of research. It is also unknown why

the perturbation of F-actin polymerization by ethanol occurs during some stages of embryonic development, but not others (Oyedele, 2013). Some have suggested a link between intracellular calcium – known to be increased in the presence of ethanol and known to be a modulator of the actin skeleton – and improper migration, but the connection has yet to be proven (Smith, 2014).

Folic acid deficiencies have been shown, independent of ethanol, to cause dysfunction of the neural tube and neural crest, leading to conditions such as spina bifida. Interestingly, supplementation with folic acid in conjunction with prenatal ethanol exposure seems to lessen its teratogenic effects (Wang, 2009). Assuredly an interesting development, it raises more questions than it answers, however. Folic acid is a necessary cofactor for nucleic acid and protein synthesis, both of which are especially necessary during early embryonic proliferation. Yet, the target for folic acid remains unclear: folic acid may upregulate proteins necessary to counteract ethanol's toxicity, or it may act on transcription factors (Xu, 2008). Folic acid also increases homocysteine levels in *Xenopus* fetuses; hyperhomocysteinemia has been independently shown to disturb migratory patterns in neural crest cells (Stickel, 2000; Shi, 2004; Brauer, 2002). Homocysteine acts on a homocysteine-sensitive, NMDA-like receptor; the exact nature of this receptor, its mechanism of action, and why it's only expressed during early embryonic stages is a topic for additional research. Some have noted, too, that renal excretion of folic acid increases following alcohol consumption, and ethanol-exposed embryos may simply be folic acid-deficient (Muldon, 1994). This, however, has not been explored substantively in the embryo, and is a likely candidate for further research.

Shh has been implicated in FASD, with ethanol exposure decreasing *Shh* levels in many animal models, and *Shh*-deficient animals sharing a phenotype similar to that of ethanol-induced holoprosencephaly (Echelard, 1993; Ahlgren, 2002; Higashiyama, 2007; Li, 2007). Higashiyama (2007) suggested that the effects of ethanol on *Shh* during a specific developmental window induce HPE, but they fail to provide a possible mechanism by which this would occur. Yelin (2007), however, found that *Xenopus* embryos treated with ethanol actually have higher concentrations of *Shh*, confounding previous results. These results were repeated by Yamada (2005) and Wentzel (2009) in mice and rats, respectively, and found no change or an increased level of *Shh*. Future studies should repeat these experiments to confirm the phylogenic conservation of ethanol-induced *Shh* decrease. Additionally, *Shh*-deficient mutants ($Shh^{-/-}$) do not express holoprosencephaly as catastrophic as severe ethanol-exposed embryos (Aoto, 2008). Yet, exogenous *Shh* does rescue neural crest cells after ethanol exposures from apoptosis, indicating that *Shh* does play a role, yet it is likely only one of many modulators (Ahlgren, 2002; Aoto, 2008).

How ethanol affects *Shh* is itself a source of debate. Ethanol has been shown in non-embryonic cells to inhibit both HMG-CoA and ACAT, enzymes involved in cholesterol metabolism (Polo, 2003). The theory is that *Shh*, which requires post-translational sterol modification, dysfunctions with altered cholesterol levels (Li, 2007). However, other researchers have found that PKA activity is increased following ethanol exposure, and causes a reciprocal drop in *Shh* (Aoto, 2008). While possible for both

mechanisms to play a significant role in disrupting *Shh* signaling, more investigation is required to confirm the authenticity of the two pathways.

Retinoic acid is the product of retinol, and is critical for modulating embryonic development, especially via the *Hox* genes (Yelin, 2004; Maden, 1996; Hall, 2009). Ethanol and retinol are both believed to be substrates for retinol dehydrogenase, which normally converts retinol into retinoic acid. Thus, ethanol may disturb the proper alignment of the axes of the neural tube and other structures. However, retinol has itself been shown to be teratogenic, disrupting normal neural crest migration. While it is clear that ethanol modulates an effect on the neural crest via inhibiting retinol dehydrogenase, it remains unclear whether the effect is due to increased levels of retinol or decreased levels of retinoic acid.

Oxidative stress has been repeatedly implicated in the pathogenesis of FASD. Davis (1990) showed that the neural crest is especially susceptible to the effects of reactive oxygen species following ethanol exposure due to lack of superoxide dismutase. Research shows that these reactive oxygen species perturb the cell membrane (Davis, 1990; Chen, 1996b) and prevent apoptosis (Wentzel, 2009). The exact mechanism of action by these free radicals is contentious. Free radicals are known to disrupt the cell membrane independently, but some have investigated the role of iron, whose conversion to ferric from ferrous iron is inhibited by ethanol (Shaw, 1998; Chen, 2000). Free iron in the cell generates additional free radicals (feeding the cycle), and causes lipid peroxidation, explaining some of the membrane damage associated with ethanol exposure (Shaw, 1999; Double, 1998). While there is evidence that iron generates free radicals,

contributes to lipid peroxidation seen in ethanol exposure, and has dysfunctional metabolism following alcohol consumption, the link between the three is presumptive, and Smith (2014) notes that additional research is necessary.

The link between genetics and prognosis following prenatal alcohol exposure is conclusive: studies comparing monozygotic and dizygotic twins have shown strong hereditary ties (Streissguth, 1993), confirmed in animal models of FASD (Debelak, 2000; Loucks, 2004). Researchers have investigated obvious genes in the metabolism of ethanol (e.g., alcohol dehydrogenase), and found a link between the more active form of the enzyme and a greater immunity to the teratogenic effects of ethanol (Debelak, 2000). Finding proper genetic ties between those who are more or less susceptible to prenatal alcohol exposure will require a much more profound understanding of the mechanisms that mediate the disease, as only then will a comprehensive list of target genes be known. Other targets, such as pdgf, may be discovered as researchers explore various genes associated with things such as cell proliferation, migration, and survival (Viljoen, 2001). The role of environmental factors – such as hormonal levels, diet, and maternal age – has not been adequately explored, and will assuredly be an area of fervent research in the coming years.

Low Dose Ethanol

More work is required to understand the role of small doses of ethanol on the developing fetus. The CDC, U.S. Surgeon General, and the American Academy of Pediatrics are all steadfast in stating that there is no safe amount or type of alcohol, or

time or consumption, which is safe for the fetus (CDC, 2015). They note that in addition to ethanol's role in FAS and FASD, prenatal alcohol exposure is conclusively linked to premature deliveries, stillbirth, miscarriage, and sudden infant death syndrome (SIDS). Animal studies also indicate grave effects of even mild ethanol concentrations, those exhibited by an average adult after one unit of alcohol.

Studies that have found no link between moderate maternal alcohol consumption and disadvantageous fetal outcomes (or have concluded that prenatal alcohol consumption at low doses can actually be beneficial) are fraught with problematic assumptions. For instance, a study by Niclasen (2013) found that children whose mothers consumed ethanol in doses up to 90 units throughout the pregnancy (roughly 2 drinks per week) exhibited better mental health at age 7. However, women in this category were significantly more likely to be educated, abstain from smoking and soda consumption, exercise, and have low BMIs; all of these factors are likely to lead to better child outcomes. While the study noted this, it failed to account for these variables in its statistical analysis and provided no evidence that, all other factors the same, moderate alcohol consumption provides any benefit. It also fails to take into account present views that ethanol affects more complex cerebral processes, and the brain at age 7 is incapable of performing this tasks in any capacity. Indeed, many symptoms of FASD don't present until pubescence.

The overwhelming body of evidence concludes that any form of prenatal ethanol exposure is universally disadvantageous. When controlling for factors like smoking and socioeconomic background, Alati (2013) found a link between maternal consumption of

the equivalent of two glasses of wine or two pints of beer with decreased cognitive and developmental outcomes by age 11. Research by Andersen (2012) found a correlation between first trimester alcohol intake and an increased risk of spontaneous abortion and stillbirth. While the increased risk in poor fetal outcomes was dose-dependent, they noted 8%, 42%, and 107% increases in fetal death following 0.5-1.5, 2-3.5, and ≥4 drinks per week, respectively.

These results are in addition to animal model studies directly showing the effect of small to moderate amounts of ethanol. While many studies utilize ethanol concentrations as high as 200 mM to ensure deleterious effects for their studies, some researchers were specifically interested in the effects of low dose ethanol. Previously, research by Czarnobaj (2014) was discussed, concluding that cranial and trunk neural crest cells exhibit decreased cellular motility and migration following ethanol at low concentrations, leading to persistent (up to 24 hours) effects on cytoskeletal remodeling and motility. These experiments were performed at ethanol concentrations at 0.5 mg/ml (10.9 mM), which is designed to emulate the blood alcohol content of a 100 lb woman after 1 unit of alcohol. These results strongly suggest a teratogenic effect of ethanol at even social drinking levels (Czarnobaj, 2014). Similar results were acquired after injection of ethanol, creating a 7 mM solution; these embryos showed defects in the neural crest and neural tube (Rovasio, 2002). While at slightly higher concentrations (26 mM, or roughly two drinks for a 100 lb woman), social levels of drinking showed decreased β-catenin expression, which as explored earlier, mediates gene expression that helps prevent apoptosis in neural crest cells (Flentke, 2011). Even more frightening

results were found by Ramanathan (1996), that ethanol concentrations as low as 1 mM caused statistically significant reductions of cell-cell adhesion due to human L1-CAM. Inhibition reached half of maximum at concentrations of 7 mM, still within the range of 1 unit of alcohol for a 100 lb woman.

These results suggest that small amounts of ethanol can have a potently teratogenic effect on the developing fetus. It also indicates that this sensitivity isn't isolated to one particular pathway: evidence exists for migratory dysfunction, β-catenin dysregulation, and decreased cellular adhesion. All signs point towards even modest amounts of ethanol exposure causing potent effects on the fetus, however significantly more research is necessary to accurately categorize its effects. While more research is assuredly needed to confirm and fully categorize the effects of small doses of ethanol, present evidence overwhelmingly suggests it has a potent effect. The vast majority of research has utilized levels of ethanol seen only in binge drinking; now that the effects of ethanol have been better discovered, elucidating whether similar effects are mediated by lower doses of ethanol is necessary.

Conclusion

Curing a disease such as FAS or FASD is extremely difficult: missing critical periods during embryonic development is nearly universally irreversible, and prevention is a much easier means of eliminating FASD. While we are discovering that some compounds – L-ascorbic acid, 5-methyltetrahydrofolate, neurotrophin-3 for example – have been shown in animal models to ameliorate some of the pathologies of the neural

crest, and some genetic strains have been shown to confer a modest resistance to the effects of ethanol, we still lack a broad enough understanding to truly discuss treatment options. Indeed, a curative treatment for FAS, FASD may never exist.

Various models for ethanol's teratogenic effects are being constructed, but they are likely not mutually exclusive; to tackle all the pathologies associated with prenatal ethanol exposure may require half a dozen different treatments. For pregnant women who have consumed alcohol, soon there may be nascent treatment options for the following day, but the medical community should be wary promoting these. It would be incredibly unwise to cast prenatal ethanol exposure as something that requires fixing, rather than something that should simply be avoided at all costs.

Because of the omnipresence of alcohol consumption in everyday life in many cultures, a complete eradication of FAS and FASD is unlikely. Even with widespread education, given that a majority of pregnancies in the United States are unplanned, social alcohol consumption may still lead to an array of children born with FAS or FASD due to their mothers consuming alcohol while unaware of their own pregnancies (CDC, 2015). Nevertheless, the persistence of FAS and FASD is disheartening, considering that it is an entirely preventable illness. The failure of Western society and many physicians to accept the overwhelming evidence that both binge and social drinking contributes to the FASD phenotype is shocking and saddening, to say the least, and may be remembered in later years as a movement not altogether different from other science deniers such as those of the anti-vaccine movement. The fundamental question when tackling the debate over moderate drinking alcohol while pregnant should be: is the small benefit yielded

from a single alcoholic drink worth the potential risk of irreversible, potentially debilitating, birth defects posed by consuming a teratogen?

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