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The Avian Embryo as a Model for Fetal Alcohol Spectrum Disorders

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Abstract

Prenatal alcohol exposure (PAE) remains a leading preventable cause of structural birth defects and permanent neurodevelopmental disability. The chick (*Gallus gallus domesticus*) is a powerful embryological research model and was possibly the first (Fere, 1895) in which alcohol's teratogenicity was demonstrated. Pharmacologically relevant alcohol exposures in the range of 20–70 mM (20–80 mg/egg) disrupt chick embryo growth, morphogenesis, and behavior, and the resulting phenotypes strongly parallel those of mammalian models. The avian embryo's direct accessibility has enabled novel insights into alcohol's teratogenic mechanisms. These include the contribution of IGF1 signaling to growth suppression, the altered flow dynamics that reshape valvuloseptal morphogenesis and mediate its cardiac teratogenicity, and the suppression of *Wnt* and *Shh* signals to disrupt neural crest migration, expansion, and survival and underlie its characteristic craniofacial deficits. The genetic diversity within commercial avian strains enabled identification of unique loci, such as ribosome biogenesis, that modify vulnerability to alcohol. This venerable research model is equally relevant for the future, as the application of technological advances including CRISPR, optogenetics, and biophotonics to the embryo's ready accessibility creates a unique model in which investigators can manipulate and monitor the embryo in real-time to investigate alcohol's actions upon cell fate.

Keywords

Fetal alcohol spectrum disorder; chicken; neural crest; cardiac development; craniofacial development

History of Chicken in Developmental Alcohol Research

The chicken (*Gallus gallus domesticus*) embryo, along with the Japanese quail (*Coturnix coturnix*), has a millennia-long history as a model for embryological study, dating back to observations by Aristotle (*Historia Animalium*, *De Generatione Animalium*) and Hippocrates (Needham 1934). Much of this reflects their ease of use. Avian embryos are enclosed in a calcium carbonate shell that is easily windowed for direct observation. Development is sustained under conditions of 37–38°C and 50–55% humidity and requires

little more than an incubator held at ambient oxygen levels. Avian embryos are more similar to mammals than are other oviparous classes because, like mammals, they possess an amnion that encircles the embryo. As with other vertebrates, many of the developmental processes that govern avian morphogenesis are conserved with mammals at the molecular and cellular levels. *Gallus gallus* remains a model of choice for embryological study and continues to contribute to our understanding of key developmental events including convergent extension, induction, cell migration, vasculogenesis, nervous system development, and epithelial-mesenchymal transformation. Avians are also popular for neurobehavioral research and directed brain manipulation during development is used to study behavioral consequences. Chicks hatch at 21 days incubation (Japanese quail at 17–18 days) and their precocial offspring can be studied soon thereafter with respect to motor, behavioral, and learning skills. Avian species can also be manipulated genetically, albeit in a limited manner.

The avian embryo is quite possibly the first model organism in which alcohol's teratogenicity was investigated. Fere (1895, 1898, 1899) and Stockard (1910, 1914) both reported that ethanol exposure (via vapor chamber) increased mortality, reduced growth, and caused embryological malformations. Patry and Ferrier (1934) showed that 5% to 10% ethanol reduced cranial flexure in explant culture, which would impair cardiac looping and septal morphogenesis. In the same year that Lemoine published his clinical description of FAS, Sandor provided the first "modern" evaluation of alcohol's developmental damage, in which gastrulation-stage exposure (64% in the air sac) impaired fundamental processes of early morphogenesis, including asymmetric blastoderm, smaller somites, reduced cranial flexure, reduced body length, and malformations of the brain, heart, otic vesicle, and caudal region (Sandor and Elias 1968). Microscopic evaluation (Sandor 1968) revealed selective damage to the neural tube including extensive necrosis, "necrospherulae sometimes coincident with mitosis" (likely apoptosis), myeloschisis (spina bifida), and cellular extrusions into the neural tube lumen. Sandor suggested that "necrosis represents a 'stereotypical answer' of the embryo to various noxae", and he proposed that alcohol is a likely human teratogen (Sandor 1968).

Mechanistic Insights from Avian Models of FASD

Subsequent research has validated Sandor's insights, and the chick embryo has been invaluable to demonstrate alcohol's damaging effects upon key developmental processes including cardiogenesis, neurogenesis, craniofacial morphogenesis, and growth. This work exploits the embryo's easy accessibility for direct observation and to perform targeted intervention during key mechanistic events. Only select processes will be discussed here.

Embryonic Growth and Nutrition

We still have a poor mechanistic understanding of why some alcohol exposures cause profound growth deficits that extend through childhood. The avian embryo is ideal to unwind alcohol's direct impact on gestational growth as it is isolated from maternal influences. A series of studies by Pennington, Shibley, and colleagues (1983 – 2001) supported that ethanol (~60 mg/egg; BACs 5–90 mg/dl on embryonic day 7; Pennington

1988) mediates this growth suppression, because co-administration of the ADH inhibitor 4-methyl-pyrazole, which would prevent oxidation of ethanol to acetaldehyde, did not normalize growth (Pennington 1988, 1990). Deficits in growth and proliferation in these embryos were associated with reductions in cAMP and protein kinase A (Pennington 1990; Boyd et al. 1984), and a parallel decline in PKC activity (Shibley et al. 1997) and selective reductions in PKC- α , - γ , and - ϵ (McIntyre et al. 1999). Addition of an anabolic agent in the form of exogenous insulin could not overcome this growth impairment (Pennington et al. 1995), and, unexpectedly, cells from alcohol-treated embryos had dramatically increased glucose uptake in response to insulin (Shibley et al. 1997; Pennington et al. 1995; Carver et al. 1999), elevated PI3 kinase activity (Shibley et al. 1997), and increased brain glucose content (Eckstein et al. 1997), perhaps indicative of alcohol-driven metabolic stress. These changes were linked to the changes in PKC activity (Shibley et al. 1997) and altered distributions of IGF1, GLUT1, GLUT3, and several IGF binding proteins (Carver et al. 1999; Lynch et al. 2001). The authors proposed these changes might reflect compensatory attempts to enhance glucose import or cellular metabolism in response to ethanol. Because gestational hyperglycemia is typically macrosomic rather than growth inhibitory, these changes might not explain alcohol-related growth suppression, but could inform other physical anomalies as hyperglycemia itself is teratogenic.

Cardiac Morphogenesis and Function

The heart is the first organ to form and function. Shortly after gastrulation, the emerging precardiac myocytes assemble to form a pair of lateral tubes that draw ventrally to fuse; the addition of cells from the secondary heart field further elongate the tube. Looping of the cardiac tube positions the four chambers and aligns the valvuloseptal cushions to form the valves and septa. Disturbances in any of these events can cause significant structural and functional heart defects, some of which are incompatible with further development. Chick is an important model to investigate mechanisms of cardiac morphogenesis and teratogenesis because of the embryo's accessibility. This work shows that alcohol exposure during gastrulation and early neurulation interferes with fusion of the bilateral tubes to produce cardia bifida, and it also interferes with the subsequent looping necessary to correctly position the atrioventricular (AV) chambers and inflow and outflow tracts (Twal and Zile 1997), as alluded by Sandor and Elias (1968). While cardia bifida and severe looping deficits are incompatible with embryo survival, more "modest" looping deficits result in severe defects such as Tetralogy of Fallot.

Basic research using the avian embryo drove the realization that cardiac functional changes affect its internal flow dynamics, and that these dynamics shape both valvuloseptal morphogenesis and wall structure. Chick is a gold-standard model to interrogate these processes in response to alcohol. Bruyere and colleagues (Fang et al. 1987) leveraged this to show that alcohol's embryo lethality during organogenesis originates from cardiovascular impairment. Exposure (158 mg/egg) during early valvuloseptal morphogenesis (HH stages 20–22) causes major cardiac defects including ventricular septal defects, failed outflow tract septation, and absent aortic arches (Fang et al. 1987); parallel ventricular and outflow tract deficits are independently described for quail (exposed at HH4, gastrulation; 16 mg per 12–14g egg, 40 mM peak alcohol level; Karunamuni et al. 2015, 2017). Using videography,

Ruckman et al. (1988) quantified dose-dependent decreases in heart rate and shortening fraction (a measure of contractility across the cycle). Bruyere and Stith (1994) further documented this hemodynamic impairment and found immediate, dose-dependent decreases in cardiac rate, stroke volume, ejection fraction, and end diastolic volume; these changes significantly reduced cardiac output for at least 10 hr following alcohol exposure (79–158 mg/egg; mean albumin level 217 ± 23 mg/dl). Using OCT (optical coherence tomography) Doppler imaging, Karunamuni et al. (2013; 2014) documented functional deficits including retrograde blood flow suggestive of abnormal AV cushions (which act as presumptive valves at this early stage) and OCT imaging revealed the cushions were indeed significantly smaller. Similar deficits in function and AV cushions were observed after neural crest cell ablation (Ma et al., 2016), and experimentally altering function using a pacing laser to mimic the increased retrograde flow led to similar morphological outcomes (Ford et al. 2017). Bruyere et al. (1994) found a reduced metabolic activity (lactate dehydrogenase; succinate dehydrogenase) within alcohol-exposed cushions that could underlie their functional impairment. Further supporting this reduced functionality are separate demonstrations that vapor chamber exposures (0.75 – 1.5 mg/l) produce myocardial wall thickening followed by increased chamber diameter, changes which suggest remodeling of the heart wall in an attempt to improve cardiac output (Kamran et al. 2013, 2016). Structural wall changes were also documented by Cavieres and Smith (2000) in alcohol-exposed hearts (50–60 mM peak embryo alcohol level) fixed at uniform contractility. The ability for real-time functional assessment of chick embryo heart shows that the structural cardiac malformations observed after gestational alcohol exposure originate, in part, from functional impairments that disrupt valvuloseptal formation and remodeling (i.e. produce valvuloseptal defects). This dysfunction limits cardiac output and thereby nutrient and oxygen availability, and thus would contribute to fetal growth deficits.

Outflow tract septation also requires the neural crest, which enable migration of myocardial populations to the forming aorta and pulmonary artery (Waldo et al. 2005). The cardiac neural crest also contributes to maturation of the cardiac conduction system (Gurjarpadhye et al. 2007) and coronary arteries (Hyer et al. 1999). Alcohol impairs neural crest migration and survival (see below) and it is often assumed these losses drive alcohol's cardiac deficits. However, a direct neural crest contribution remains surprisingly untested apart from one report that alcohol exposures which disrupt facial morphogenesis do not reduce neural crest occupation of the outflow tract despite the presence of heart defects (Cavieres and Smith 2000). This is consistent with Kirby's observations that the neural crest effect on cardiac function through abnormal signaling to the secondary heart field may be more important for heart function than the presence of neural crest derivatives in the outflow tract. The lack of effect on neural crest inhabitation of the outflow tract might be that the cardiac neural crest has greater developmental plasticity than does the cranial neural crest, and thus a greater ability to compensate for losses. Another possibility is that the timing of vulnerability differs, so that a single exposure may affect one neural crest population and not the other. The precise role – if any – for cardiac neural crest in alcohol-mediated cardiac deficits requires additional study.

Taken together, work in avian embryos clearly documents the mechanistic role played by cardiac flow deficits in causing alcohol-related heart defects (Bruyere 1994), a conclusion

supported by the elegant OCT imaging of alcohol-exposed quail by Watanabe's team (Karunamuni et al. 2014; Peterson et al. 2017), and by echocardiographic imaging in the alcohol-exposed fetal mouse (Serrano et al. 2010).

Nervous System Development and Function

The chick embryo central nervous system (CNS) has been a powerful model to investigate neuronal outgrowth and differentiation since Nobel laureate Rita Levi-Montalcini's pioneering work identified nerve growth factor (NGF). The chick and mammalian CNS share similar responses to alcohol. Brain weights are reduced, as is cellularity for select populations such as cerebellar Purkinje cells (Swanson et al. 1994). Like mammals, alcohol-exposed chick (6.3 mg as 40% alcohol directly atop the blastodisc) displays significant apoptosis in the early brain and spinal cord (E2), and this is accompanied by reduced proliferation and cell extrusions into the neural tube lumen (as per Sandor; Giles et al. 2008). Neuronal migration is similarly impaired, and their neurite extensions are fewer, shorter, and more disorganized relative to the central body and adjacent cell layers (Dow and Riopelle 1985; Kentroti and Vernadakis 1991a; Quesada et al. 1990). Using the classic chick spinal cord explant, Dow and Riopelle (1985) showed that alcohol muted neurite outgrowth in response to exogenous NGF (TD50 100–170 mg%); this was not due to impaired NGF binding but to reductions in neurotrophic factor production. Heaton et al. (1992; 24 mg/d E4-E13, 108 ± 25 mg/dl at E16) independently confirmed these reductions, and provision of neurotrophic factors compensated for these impairments (Heaton and Bradley 1994). Thus, chick displays similar reductions in neuron migration and process formation as seen in mammalian models of FASD.

Work in chick also informed the stage-dependence of alcohol's effects on neuronal fate and differentiation. For example, alcohol exposure at E1-2 (50 mM in culture; 45 mg/egg) reduces choline acetyltransferase (ChAT) and increases tyrosine hydroxylase and glutamate decarboxylase activity (Brodie and Vernadakis 1990; Kentroti and Vernadakis 1990, 1991b), whereas the reciprocal outcome was obtained when exposure occurred at E8 (Lee et al. 1992). Reductions in ChAT persist in the alcohol-exposed forebrain and optic tectum (Swanson et al. 1994), consistent with the cholinergic reductions seen in mammalian PAE models.

The avian eye's exceptional size and accessibility makes it an excellent model to investigate the ocular anomalies associated with FASD. Chick studies show that alcohol damages retina and optic nerve development. The optic nerve is significantly smaller and contains far fewer myelinated axons, and the myelin that is present is disorganized and thinned (Chmielewski et al. 1997; Tufan et al. 2007). The retina is disorganized, and development of all three major cell layers (ganglion cell, inner plexiform, pigment epithelium) is impaired, with fewer synapses and gap junctions and increased cellular degeneration. Alcohol exposure during retinal synaptogenesis led to retinal populations with increased GABA (but not aspartate) release in response to glutamate but no changes in GABA-positive cell numbers (Pohl-Guimaraes et al. 2010).

Avian species including chick are classic models for learning. Chick studies were possibly the first to show that developmental alcohol exposure impaired select behaviors including

associative learning, maze finding, reversal learning, and acquisition of fear (Fletcher et al. 1916). More recently, avian studies show alcohol exposure (20 – 80 mg/egg) causes behavioral impairments in domains similar to those in other FASD animal models including long-term memory formation (Rao and Chaudhuri 2007), conditioned learning (Linakis and Cunningham, 1980), detour learning (Means et al. 1988, 1989), attention to maternal auditory signals (Heaton and Paiva 1993), reflexive motor activities such as egg pipping (Means et al. 1989) and wing-flap (Smith et al. 2011), and heightened fearfulness (Smith et al. 2011). This strong conservation of behavioral impairments across vertebrate orders emphasizes the vulnerability of *Homo sapiens* to alcohol's teratogenicity.

Craniofacial Morphogenesis

A hallmark of prenatal alcohol exposure are facial anomalies that include epicanthal folds, thin upper lip, and absent/reduced philtrum (Astley and Clarren 2001). It seems surprising that avian species are good models to study alcohol and facial development because they lack these soft tissue structures. However, skeletal muscle shape and position is informed by the underlying bone and cartilage, as exemplified in the success of forensic reconstruction. And, indeed, the alcohol-exposed chick exhibits the same underlying dysmorphologies as documented in human FASD (Suttie et al. 2013) including reduced midface, hypotelorism, and micrognathia (Cartwright and Smith 1995b, Rovasio and Battiato 2002; Su et al. 2001). As with the heart, the chick embryo is a powerful and popular model that has elucidated much of what we know about craniofacial morphogenesis and particularly the cranial neural crest, which is a pluripotent stem cell population that forms the facial bone and cartilage (but not the skull bones), cranial nerve elements, and the stroma of thoracic structures (i.e. thymus, outflow tract) (Dupin and Le Douarin, 2014). The physical accessibility of the chick facial anlage combined with the genetic power of the mouse has substantially advanced the understanding of how alcohol alters facial development.

Mechanisms of craniofacial morphogenesis are highly conserved across vertebrates from human to zebrafish. These mechanisms are the focus of several recent reviews (Dupin and Le Douarin, 2014; Simões-Costa and Bronner, 2015) and readers seeking additional detail are directed to those. To summarize, the face is essentially derived from the brain and, thus, facial length and width partly reflects the underlying brain size (Marcucio et al. 2015). Its initial dimensions are approximated shortly after gastrulation with the rostral emergence of the prechordal plate, which induces the midline neural plate in the overlying ectoderm. Neural crest progenitors are specified at the neural plate's lateral margins, and thus their overall population size is influenced by the prechordal and neural plate dimensions. Work in *Xenopus*, zebrafish, and mouse document that gastrulation-stage alcohol exposure impairs prechordal plate extension and neural induction, in part through repression in sonic hedgehog (SHH) (Aoto et al. 2008; Blader and Strahle 1998; Kietzman et al. 2014; Nakatsuji et al. 1983). Elegant imaging work in mouse established how these changes reduce brain and therefore facial size (Lipinski et al. 2012). This failure to expand the anterior prechordal plate contributes to the holoprosencephalic aspects of the facial phenotype in FASD. Neural crest progenitors are induced immediately lateral to the neural plate, and thus a smaller prechordal and neural plate would also reduce the neural crest population size. Work in chick supports this mechanism, as gastrulation-stage exposure (50 mM, 20 mg/egg)

reduces the expression of early neural crest markers including *foxd3*, *wnt6* and *snai2* (Flentke et al. 2011), suggesting their suppressed induction and/or population shortfall.

As the elevated neural folds fuse dorsally to form the neural tube, the neighboring neural crest cells undergo an epithelial-mesenchymal transformation wherein they delaminate and emigrate ventrolaterally to populate the facial primordia and the branchial arches that form upper thoracic structures such as the outflow tract and thymic epithelium. Not all neural crest cells make this journey and a select subset are deleted at this time through programmed apoptosis; work in chick shows these “unwanted” cells form, for example, novel muscle/bone attachment sites in the jaw, and thus they may be an evolutionary legacy to create facial plasticity (Ellies et al. 2002). Sulik’s pioneering work in mouse (Sulik et al. 1981; Kotch and Sulik 1992) shows that alcohol expands these cell death-enriched domains – as observed earlier by Sandor (1968) – and correctly proposed that the facial anomalies in FASD represent a neurocristopathy. Using antibody resources unique at the time to chick, Smith and colleagues (50 mM, 20 mg/egg for 2hr) showed these cellular losses include HNK-1⁺ neural crest cells, and this degeneration largely occurs at a specific developmental time coincident with their migratory emergence (Cartwright et al. 1995b) and mirroring the timing described for mouse (Kotch and Sulik 1992). Taking advantage of the chick embryo’s direct accessibility, those investigators applied small molecules to show this cell death represents caspase-dependent apoptosis within neural crest and neural tube (Cartwright et al. 1998). Follow-up studies using genetic and small molecule approaches deciphered that the signaling mechanism underlying this alcohol-mediated apoptosis involve calcium-mediated Wnt signaling (Figure 1). Specifically, exposure of early neuroprogenitors including neural crest to pharmacologically-relevant alcohol levels (10 to 100 mM) invokes a calcium transient (EC₅₀ = 52 mM), similar to its effect on oocytes, gastrulating mouse embryos, and placental trophoblasts (Debelak-Kragtorp et al. 2003; Garic-Stankovic et al. 2006). This calcium transient originates from a pertussis toxin-sensitive G protein-coupled receptor (Gα_{i/o}) and inositol-phosphate signaling in a dose-dependent manner (Garic-Stankovic et al. 2005) and activates the calmodulin-dependent protein kinase CaMKII (Garic et al. 2011); blockade of these steps using either small-molecule or targeted misexpression approaches normalizes neural crest survival in alcohol’s presence. Inhibition of other calcium-dependent kinases including protein kinase C and CaMKIV was ineffectual against alcohol, showing the pathway’s specificity. Ellies et al. (2000) previously showed that the programmed cell death within a subset of neural crest progenitors depends on a non-canonical Wnt effector, and, following this lead, Flentke et al. (2011) found that alcohol destabilizes β-catenin and its Wnt-dependent transcriptional activity within neural crest cells. The β-catenin destabilization is calcium-dependent and mediated not by known Wnt effectors including GSK3β and calpain, but rather by CaMKII (Flentke et al. 2014). They went on to identify three previously-unrecognized CaMKII phosphorylation sites within β-catenin that target the protein for proteolytic destruction. Overexpression of β-catenin or dominant-negative CaMKII prevents the alcohol-mediated apoptosis, whereas truncated TCF1 or constitutively-active CaMKII phenocopies alcohol’s proapoptotic effects. It is currently unclear if this reflects a direct activation of calcium-Wnt signaling by alcohol, or if instead alcohol activates distinct signals which converge upon that pathway.

An additional contribution to alcohol-mediated neural crest death may be from oxidative damage. Neural crest has inherently low expression of superoxide dismutase (SOD), and exogenous SOD protects these cells from alcohol-induced apoptosis (34 mM in culture) and reactive oxygen species generation (Davis et al. 1990). Exogenous antioxidants confer a similar protection to alcohol-treated mouse neural crest (50 – 100 mM; Chen and Sulik, 1996). Trophic signals in addition to canonical Wnt signals also support the survival of alcohol-treated neural crest. The cytokine Neurotrophin-3 (but not Ciliary Neurotrophic Factor) promotes neural crest proliferation and survival in response to alcohol (100 mM for 3hr; Jaurena et al. 2011); neural crest cells express the Trk-C receptor, but whether this represents repletion of absent neurotrophin or generalized trophic support was not tested. Alcohol also downregulates expression of SHH in a range of vertebrate models and, in chick (1% alcohol applied directly), exogenous SHH enhances neural crest survival within the facial primordia (Ahlgren et al. 2002).

Alcohol-exposed neural crest cells that do not apoptose instead migrate through the facial mesenchyme to occupy the branchial and aortic arches, where they differentiate into the appropriate lineage (chondrocyte, neuronal, melanocyte); their fate is dictated both by their timing of emigration and growth factors within their external environment. Extensive research, again predominantly in chick but also in zebrafish, demonstrates that alcohol inhibits this migration in a dose-dependent manner. At high alcohol levels (150mM), migrating cells travel shorter distances and exhibit disorganized actin and fewer filopodia, lamellipodia, and focal adhesions (Rovasio and Battiato 1995, 2002). Although many cells follow their correct lateral routes, others do not emigrate, and still others enter the neural tube lumen (Cartwright and Smith 1995b; Rovasio and Battiato 2002). At lower alcohol concentrations (34–68 mM) the converse occurs and alcohol enhances the migratory distances (Oyedele and Kramer 2013). Along similar lines, we find that transient alcohol exposure (52 mM, 2hr) enhances neural crest migration due to the alcohol-mediated upregulation of *Snai2*, which initiates epithelial/mesenchymal transformation and migratory capacity (Flentke et al., submitted). Neural crest exposed to high alcohol concentrations (150–172 mM) have fewer lamellipodia and filopodia, a disorganized cytoskeleton, and reduced migratory capacity (Rovasio and Battiato, 2002), changes quite similar to those described for alcohol-exposed gastrulation-stage mesodermal (Sanders et al. 1987). Further mechanistic insight comes from an elegant study by Tolosa et al. (2016) in which SHH produced within the forebrain basal plate and optic stalk orients a subset of cranial neural crest migrants toward the optic stalk. Alcohol (100 mM) inhibits directed migration toward the SHH source, in part by reducing SHH expression (Ahlgren et al. 2002; Tolosa et al. 2016). Alcohol (50 – 100 mM in culture) also reduces neural crest proliferation (Jaurena et al. 2011; Flentke et al. submitted) and promotes their precocious differentiation in micromass culture of facial primordia (171 mM; Hoffman and Kulyk, 1999), and these changes would further limit the growth and expansion of developing craniofacial structures.

Genetic Influences on Alcohol Responses

Although we currently lack a consistent technology for germline genetic manipulation of avian species, intensive breeding within the poultry industry has generated genetic lines that offer useful insights into alcohol's actions and underlying mechanisms. Human twin studies

initially suggested that genetics might influence outcomes in alcohol-exposed pregnancies (Streissguth and Dehaene 1993). Using chick, Bruyere and Stith (1993) were among the first with any model to demonstrate that the embryo's genetic background modifies vulnerability to alcohol (78 mg/egg), showing that Hy-Vac SPF type L had a four-fold to ten-fold increase in ventricular septal defects compared with Hy-Vac SPF type V and DeKalb Delta strains. Bupp-Becker and Shibley (1998) similarly found genetic influences upon alcohol-mediated growth suppression (42 – 78 mg/egg), and two broiler strains (Peterson x Hubbard; Ross x HY) had greater growth reductions in response to graded alcohol exposures compared with two layer strains (DeKalb Gold, White Leghorn unspecified); this may reflect that broilers are under intense genetic selection for rapid growth and muscle accumulation, whereas layer strain selection emphasizes early sex dimorphism and egg production. These two broiler strains also showed greater growth reductions in response to acetaldehyde than did layer W36 Ginther White Leghorn (Hartl and Shibley 2002). DeKalb Gold had greater resistance to alcohol lethality than the other strains (Bupp-Becker and Shibley 1998), work that was later mirrored in our group (50 mM for 2-3hr, 20 mg/egg) as DeKalb strains were less vulnerable to heart defects (Cavieres and Smith, 1999), neural crest death (Debelak and Smith 2000), and facial anomalies (Su et al. 2001). The pattern of and vulnerability to neural crest apoptosis is also a function of the embryo's genetic background, and these differences were not due to shifted timing of apoptosis or differential alcohol exposure (Debelak and Smith 2000). Genetics also affect the fine details of craniofacial shape and these could be categorized as wider/flattened, hypoplastic, or unaffected (Su et al. 2001). Myocardial phenotype is also modulated by the embryo's genetic background. This collective work endorses that chick strain must always be reported in alcohol research.

Taking advantage of these differences in vulnerability, our research group characterized the transcriptome of cranial neural folds in alcohol-sensitive and alcohol-resistant chick strains, both in alcohol's absence and 6hr after alcohol challenge (52mM for 2-3hr). Using whole exome sequencing, we found that the greatest gene-level changes in response to alcohol were significant reductions in ribosome, oxidative phosphorylation, and spliceosome pathways (Berres et al. 2017). Ribosome dysbiogenesis causes neural crest apoptosis and craniofacial deficits in humans and in animal models (Danilova and Gazda 2015; Yelick and Trainor, 2015), including Diamond-Blackfan anemia and Treacher-Collins syndrome. To test the potential contribution of expression-level differences to alcohol vulnerability, we used a morpholino approach in zebrafish to create targeted reductions in RPL5A, RPL11, or RPS3A, but to intermediate levels that did not affect facial development. Addition of a moderate alcohol dose produces significant craniofacial deficits and neural crest apoptosis only in embryos that are also haploinsufficient for RPL5A, RPL11, or RPS3A (Berres et al. 2017). This supports work in zebrafish and mouse showing that otherwise silent genetic alterations increase vulnerability to alcohol's teratogenesis.

Interestingly, these same three KEGG pathways – ribosome, oxidative phosphorylation, and spliceosome – also had the greatest gene-level differences in a whole-exome comparison of untreated cranial neural folds isolated from closely-related chick strains that were either vulnerable or resistant to alcohol-induced neural crest apoptosis (Garic et al. 2014). Because ribosome biogenesis occupies a substantial portion of the cellular energy budget (70%-80%, Warner et al. 2001), cells monitor nucleolar stress and link it to p53 activation during periods

of cellular stress (Danilova and Gazda, 2015). Current work in our lab investigates how alcohol exposure leads to nucleolar stress and ribosome dysbiogenesis. Human ribosomopathies are otherwise compatible with life and some affected individuals display facial anomalies akin to those of FASD (Yelick and Trainor 2015), suggesting this gene pathway contributes to the variable outcomes in alcohol-exposed pregnancies.

Experimental Factors for Avian Models of Developmental Alcohol Exposure

Here, we discuss broad principles and advantages/disadvantages of using avian embryos for alcohol research. Specific protocols on this topic can be found in Smith et al. (2012). Fertile eggs are readily procured from local hatcheries at modest cost and are suitable for most research. Store fertile eggs at 15°C if not used immediately; storage at 4°C is lethal. Embryo development is temperature-driven, thus eggs must be experimentally blocked from random incubator locations even under forced air. Other advantages of the model include its ease of care, low startup, and a non-onerous ACUC if only embryos are involved. Its single greatest limitation, of course, is the inability to capture maternal contributions to FASD. Such contributions include maternal metabolism of alcohol and acetaldehyde, nutritional factors, changes in metabolic flux, placental dysregulation, maternofetal circulation, and the like. Conversely, it offers the capability to isolate alcohol's direct effects upon the embryo.

A constellation of exposure routes and incubation methods are available. *In ovo* exposure most closely mimics normal development and poses the least stress. Exposure volumes must be smaller than the air sac volume (<300 µl) so as not to crush the embryo, and the shell/air sac must be perforated to displace the air. We developed a method (Smith et al. 2012) that injects alcohol – diluted in isotonic saline – through a tiny hole poked through the blunt end and air sac of a horizontally held egg, and into the yolk center using a calibrated needle and glass microliter syringe. The specific gravity of yolk is 1.035, slightly greater than both water and water/alcohol mixtures; thus, aqueous yolk injections rise to contact the overlying embryo (Walker 1967; Cartwright et al. 1998). This generates highly reproducible exposure kinetics (Figure 2A; Cartwright et al. 1998; Debelak and Smith 2000) with peak exposure for 10-90min post-injection; levels equilibrate throughout embryo, white and yolk by 2-3hr thereafter. Exposure via air sac injection, typically directly over the embryo (Bruyere and Kapil, 1990; Koda et al. 1980; Sandor and Elias, 1968) has similar kinetics that peak within 10min of injection and parallel those of yolk injection (Figure 2B). Vapor exposure at 0.75–1.5 mg/l in a modified incubator impairs chick development, but embryo alcohol levels were not quantified (Kamran et al. 2009). Shell-less incubation (*ex ovo*) uses variants of New culture, wherein the embryo – still attached to the vitelline membrane and area opaca – is explanted atop a substrate such as 50/50 agar/egg white and incubated as per cell culture (Auerbach et al. 1974; Chapman et al. 2001; Hornbruch 2008). This approach permits time-lapse assessment and direct intervention (i.e. bead implant, laser pacing, etc). Eggs can also be windowed for precise staging (Hamburger and Hamilton, 1951) and for direct manipulation, such as the electroporation of expression constructions, or the application of small molecules using microbead delivery systems (i.e. Garic et al. 2005). The window is sealed using leak-proof Scotch 3M black electrical tape.

With respect to dose, embryo levels should be pharmacologically relevant and no higher than ~250mg% (~0.25% or 54mM). Direct application of alcohol to the embryo within a windowed egg should never be used due to osmotic effects that cause non-specific damage. Chick embryos have little-to-no alcohol metabolism until day nine of incubation (Wilson et al. 1984). Chick liver ADH has a K_m of 0.10 to 0.52 mmol and V_{max} of 1.15 ± 0.3 $\mu\text{mol}/\text{min}/\text{g}$ (Sinclair et al. 1990). Studies using the ADH inhibitor pyrazole affirmed ethanol as the proximate teratogen (Gilani et al. 1986). The egg shell is weakly porous to alcohol and losses average ~5% daily until day 11, when liver metabolism rapidly clears the remainder (Wilson et al. 1984). Thus, chick typically represents a chronic binge model.

Despite years of effort, the single major technical limitation is the relative resistance of avian species to germline modification. This may reflect the difficulty in identifying germinal centers since eggs are laid just prior to the blastula stage (~4000 cells). There are irregular reports of transgenic quail embryos (reflecting their easier husbandry) created through lentiviral vectors. Many feature expression of fluorescent reporters (i.e. eGFP, mCherry) driven by ubiquitous or cell-specific promoters for cell tracking studies (Chapman et al. 2005; McGrew et al. 2004; Huss et al. 2015; Park and Han, 2012; Poynter et al. 2009). CRISPR technology to target ova and sperm may finally make germline knockdown feasible.

Where the avian embryo excels for FASD research is in mechanistic studies of dysmorphology. It has been especially powerful for morphogenetic dissection of structures including craniofacial, cardiac, limb, early brain, and the vasculature. Annotation of the chicken genome, sequenced in 2004, is robust and affirmed that gene homologies with mammals are highly conserved. Expression vectors work robustly in chick (apart from the thymidylate kinase promoter). Expression is readily manipulated using *in ovo* microinjection or electroporation of reporter plasmids, retrovirus constructs, morpholinos, and siRNA (Nakamura et al. 2004; Flentke et al. 2011), and most mammalian constructs produce equivalent outcomes in chick.

Final Thoughts and Future Perspectives

In summary, the chick embryo may be the model in longest use for the study of alcohol's developmental damage. It forms a strong quartet with mouse, zebrafish, and *Xenopus* to investigate alcohol's morphogenetic and molecular mechanisms within the embryo, and is especially superb for investigations into the neural crest and face, heart, and spinal cord. The embryo's accessibility for real-time physiological assessment led to the novel insight that alcohol is a cardiac teratogen because it disrupts the blood flow dynamics that drive chamberization and alignment of the inflow and outflow tracts. Its history as an early model of neural crest development permitted demonstration that alcohol caused neural crest losses, and elucidated the calcium-dependent signals that underlie that apoptosis. Recent technological innovations continue to enhance the model's utility for FASD research. CRISPR technology renders avian species amenable to germline-level genetic manipulation to create knockouts and transgenics that can interrogate alcohol's mechanism. Transgenic reporter quail lines now exist and, because the embryos are ideal for time-lapse imaging, investigators can follow alcohol's actions upon cell fate in real-time. The continued growth

of its genomic characterization facilitates the identification of novel genes and epigenetic marks through which alcohol disrupts embryogenesis. The simplicity of whole-mount culture for extended times especially lends avian embryos to biophotonic approaches, such as optogenetics, that manipulate cell fate and gene expression in real-time, and these will be excellent models to investigate, for example, alcohol's impact upon neural migration and connectivity while minimizing the need for complex surgery. The model also lends itself to functional testing of gene candidates and mechanisms with the same rapidity as zebrafish or *Xenopus*. It has been a valuable model for 122 years and we raise a (non-alcoholic) toast to its continued success in the century to come.

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Figure 1. Calcium-dependent mechanism of apoptosis in alcohol-exposed cranial neural crest
 Panel summarizes multiple studies from this lab, as reviewed in text. Chick embryos of the three somite stage are exposed to alcohol (EC50 = 52mM) and display an intracellular calcium transient within 2–3 seconds of exposure, as detected using the ratiometric intracellular calcium indicator Fura-2. Box indicates the imaged region, comprised of neural folds including neural crest progenitors. Asterisk (*) indicates the increased Fura-2 (green) calcium signal. Within 1 minute of alcohol addition, increased phospho-CaMKII protein is enriched within neural folds as detected using whole-mount immunohistochemistry (compare green signal at arrows). Two hours thereafter, nuclear β -catenin protein is lost from the dorsal neural folds including the boxed region enriched in premigratory neural crest; inset shows the nuclear loss at higher magnification (compare green signal at arrows; blue are DAPI-stained nuclei). Twenty hours after alcohol exposure, apoptotic neural crest cells (arrows) are observed as visualized by double-staining for the neural crest marker Sox 9 (green) and LysoTracker Red (red), which we showed previously detects TUNEL+ cells in this model. Adapted from Smith et al. 2014. m, mesendoderm, nc, neural crest; nf, neural folds.

Figure 2. Comparison of *in ovo* alcohol exposure kinetics in two different exposure routes
(A) Embryo alcohol content following administration of 20 mg (20 μ l of 100% ethanol) in a total volume of 250 μ l, injected into the yolk center via microliter syringe. Ethanol levels reach homeostasis by 120 min after injection. **(B)** Embryo alcohol content following administration of 26 mg (33 μ l of 100% ethanol) in a total volume of 160 μ l, injected into the air sac via microliter syringe. Ethanol levels do not reach homeostasis until 24 hours post-injection. The two routes give similar peak ethanol exposures, but the yolk route's area-under-the-curve resembles a binge exposure, while the air sac route resembles chronic exposure. (A) is adapted from Debelak and Smith (2000). (B) is adapted from Koda et al. 1980. Values are mean $\pm$ SEM.