A Comprehensive Comparison of Teratogenic Compounds Known to Induce Neural Tube Defects in the Chicken Embryo

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A Comprehensive Comparison of Teratogenic Compounds Known to Induce Neural Tube Defects in the Chicken Embryo

Micah Marie Ross

A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

A Comprehensive Comparison of Teratogenic Compound Known to Induce Neural Tube Defects in the Chicken Embryo

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Doctor of Philosophy

One of the first embryonic structures generated during early human development is the neural tube. The embryonic process of neurulation, including neural tube closure, is necessary for proper brain and spinal cord development, whereas improper closure leads to neural tube defects including anencephaly, spina bifida, and craniorachischisis. The mechanism by which these defects occur is unknown, but some evidence suggest that redox disruption may play a role. Cellular redox state is important in regulating key processes during neural tube closure, including differentiation, proliferation, gene expression, and apoptosis. This study aims to determine whether redox potential shifts and these key processes are affected similarly or differentially after treatment with three neural tube defect-inducing developmental toxicants: ceramide (C2), valproic acid (VPA), and fumonisin (FB1).

Using the P19 cell model of neurogenesis, in both undifferentiated and terminally differentiated cells, we analyzed glutathione (GSH) redox (Eh) potential to evaluate the effect of each toxicant over time. We show that in C2 and VPA treated cultures an oxidizing shift occurs, but interestingly, FB1 treatment results in a reducing shift in embryonic GSH Eh as compared to untreated cultures. Using the chick embryo model, comparable redox shifts were observed as were seen in P19 cells, supporting similarity between the models. To better understand how differential shifts in the redox state can result in similar defects, we then examined potential variances in neuronal differentiation and cellular proliferation, survival, metabolism, adhesion, and gene expression under each treatment. We report changes to cellular and embryonic endpoints that support dysmorphogenesis, likely the result of oxidizing or reducing stress that altered redox state.

These results support the need for broad comparative analyses such as this to determine whether toxicants that cause the same types of defects, whether NTDs or others, act through similar or different mechanisms. This can better inform preventative measures used to reduce the risk and occurrence of birth defects.

Key Words: ceramide, fumonisin b1, valproic acid, neural tube defects, teratogen, maternal obesity, birth defects, oxidative stress, chicken embryo
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CHAPTER 1: Introduction

Neural Tube Closure and Defects

During the initial stages of development, the embryo begins as a flat sheet of cells (Figure 1.1 A). Intracellular signaling within this primitive tissue initiates morphological changes, leading to bilateral elevation and bending of the left and right sides of this sheet, parallel to the notochord, forming the neural groove and neural folds (Figure 1.1 B). These folds will initially meet at the dorsal side of the future hindbrain of the developing embryo and adhere together. Subsequent adhesion extends both anteriorly and posteriorly along the axis of the embryo, resulting in an enclosed tube by day 28 of development (Figure 1.1 C). This early structure, known as the neural tube (NT), gives rise to the brain and spinal cord of the embryo. If the neural folds fail to rise, bend, or adhere at any point along the axis during this process, the resulting opening gives rise to a neural tube defect (NTD; Figure 1.1 D).

Neural tube defects (NTDs), the second most common among birth defects, reportedly affect 0.03-19.94/1000 pregnancies worldwide depending on location and reporting methods (1991; Mitchell, 2005; Copp et al., 2013; Li et al., 2016; Zaganjor et al., 2016). One recent review indicated that rates vary significantly, reporting ranges in African regions from 5.2-75.4/10000, in European countries from 1.3-39.5/10000 births, and in the Americas from 1.4-27.9/10000 (Bhandari and Thada, 2020). The type and severity of NTDs depends upon the location and extent of the defect (Figure 1.2). Defects, defined by the location along the anterior-posterior axis, include cranial, caudal, and complete NTDs. Cranial NTDs include anencephaly (Figure 1.2 A) and encephalocele (Figure 1.2 B). Anencephaly results in partial or complete failure of brain and skull development, occurring in 1/4,600 births in the United States each year (Mai et al., 2019). Nearly all babies affected by anencephaly die in utero or shortly
Figure 1.1: Neural Tube Closure.
(A) The early embryo begins as a flat sheet of cells. The ectoderm of the embryo is divided into three cell types: neural ectoderm (green), neural crest progenitors (white), and non-neural ectoderm (yellow). Ventral to the embryo is the notochord (dark blue circle), a key signaling center in early development. (B) During neurulation, mechanical forces a) drive lateral cells toward the midline of the embryo (yellow and white arrows) and b) force cells dorsally near the midline (green arrows). (C) Final stages of neurulation require dissociation and subsequent adhesion of neural (green) and non-neural (yellow) cells to form the neural tube (green) and overlying ectoderm (yellow). Neural crest cells migrate ventro-laterally (white). (D) Failure of any mechanical changes, cell signaling, adhesion, or cell shape changes result in an opening in the neural tube.
after birth (Bakaniene et al., 2016). Encephalocele is a rarer defect, affecting 1/10,500 babies in the United States annually (Mai et al., 2019). Unlike anencephaly, encephalocele is an enclosed, sac-like protrusion, containing projections of the brain and membranes. This sac results from an opening in the neural tube anywhere from the back of the skull to the nose of affected babies. Surgical interventions are required to correct these defects, and long-term effects are dependent upon the location and severity of the protrusion (Siffel et al., 2003).

Spinal NTDs are defined as open or closed dysraphism (failure of neural tube closure). Closed NTDs are known as spina bifida occulta, or “hidden,” indicating that these defects are subcutaneous or below the skin (Figure 1.2 C). The vertebrae, most commonly S1, S2 or both, fail to form completely around the spinal cord, and generally cause no physical problems for the affected individual. Spina bifida occulta may go undetected in 10-20% of the general population, except for a tuft of hair at the base of the spinal cord in some cases. Open dysraphism defects are more detrimental to infant health and mortality, and include myelocele, meningocele, and myelomeningocele (Figure 1.2 D). Myelocele is exposed spinal tissue without skin or meninges, the membranes that enclose the spinal cord and brain, at an opening at the base of the back. In meningocele, the meninges protrude through the vertebrae and skin in a spinal fluid filled sac. Infants born with meningocele may exhibit no symptoms, experience bladder and bowel dysfunction, or display lower-limb paralysis. Myelomeningocele is a compounded defect wherein the spinal fluid-filled sac at the base of the spine contains nerves and part of the spinal cord. Myelomeningocele is the most severe type of open NTDs, resulting in babies who suffer from mild to severe disabilities throughout their lives, including incontinence and partial or complete paralysis (Bakaniene et al., 2016). Chiari II malformation of the hindbrain and resulting
Figure 1.2: Types of Neural Tube Defects in Infants.
(A) Anencephaly. (B) Encephalocele. (C) Spina bifida occulta. (D) Open spina bifida. (E) Craniorachischisis.
hydrocephalus are associated with nearly 90% of open spinal NTDs (van Gool et al., 2018), often resulting in additional surgeries and health concerns for affected children and adults. In some rare cases embryos may develop craniorachischisis, a complete failure of neural tube closure (Figure 1.2 E). Survival is impossible in cases of craniorachischisis, and medical termination is recommended.

Maternal Obesity

Maternal obesity has increased more than 70% in the last decade (Rasmussen et al., 2008; Jarvie and Ramsay, 2010). From 1980-2013, the number of adult, child-bearing women who were overweight, increased nearly 18% to 38% (Ng et al., 2014). An elevated BMI during pregnancy puts overweight and obese mothers at greater risk for pregnancy-related disorders such as hypertension, preeclampsia, and gestational diabetes (Chen et al., 2020). Obesity is a co-morbidity that is associated with a 2-fold increased risk for the development of NTDs (Rasmussen et al., 2008; Huda et al., 2010). Type 2 diabetes mellitus (T2DM) is another metabolic pathology linked to obesity and can increase the risk of NTDs in offspring by 2-fold (Ray et al., 2007). Hyperglycemia, a condition arising from insulin resistance present in T2DM and often obesity, has been studied extensively and is known to generate oxidative stress and reduced expression of Pax3, a transcription factor found to induce ubiquitination and degradation of p53 in the neural tube (Chappell et al., 2009; Wang et al., 2011). While insulin resistance and hyperglycemic conditions are often associated with obesity, it is important to note that multiple studies have shown that the effects of obesity and diabetes both independently and synergistically affect fetal development (Bo et al., 2003; Simmons, 2011; Racusin et al., 2012). Therefore, other biological factors that play a role in metabolic syndrome, including elevated free fatty acids and inflammatory cytokines as well as altered hormone profiles, must be
investigated. Studying the ceramide axis and its role in NTD formation is one important area that needs further investigation.

Environmental Causation of Embryo Defects

Environmental factors have been a central focus of NTD research due to the potential to improve fetal outcomes through intervention. The maternal environment is critical during fetal development; obesity, cigarette use, diabetes, hyperthermia and stress have all shown to have negative effects on fetal growth, health, and mortality (Qiu et al., 2013; Alexander et al., 2015; Gaillard, 2015; Ross et al., 2015). Recent years have revealed maternal diet influences embryonic development. For example, a cluster of infants were born with NTDs in Cameron County, Texas between 1990-1991. In one alarming 36-hour period, 3 anencephalic infants were delivered in one hospital, with 3 additional cases over the next 6 weeks. Overall, the rate of NTDs along the Texas-Mexico border increased to 2.7/1000 live births compared to the normal rate of 0.2/1000 at that time in the United States (Missmer et al., 2006; Seidahmed et al., 2014). As health officials investigated the potential causes for this increase in NTDs, they discovered a link between this localized human outbreak and a nationwide epidemic of equine leukoencephalomalacia (ELEM) just one year prior. Scientists tied the ELEM outbreak to a mycotoxin produced by a common corn mold known as fumonisin (Missmer et al., 2006; Reisinger et al., 2016). While corn was removed as a common horse feed, humans continued to consume the crop. Studies showed higher than normal fumonisin concentrations in the corn meal consumed by women in this area, suggesting a causative link between fumonisin B1 (FB1), a mycotoxin produced by the Fusarium verticillioides mold, intake and NTDs (Hendricks, 1999; Missmer et al., 2006; Voss et al., 2006). Maternal FB1 exposure, most commonly associated with consumption of contaminated masa and rice, also induces NTDs in mouse models,
suggesting vertebrate neuronal development and health are affected by FB1 (Gelineau-van Waes et al., 2009; Liao et al., 2014). It is unclear whether folate supplementation can rescue embryos from FB1-induced defects.

Antiepileptic Pharmaceutical Associated Neural Tube Defects

More than 500,000 women of child-bearing age in the United States have epilepsy, a disorder in which she experiences recurring, unprovoked seizures (Hirtz et al., 2007). Epilepsy carries risks for several pregnancy-related complications, including preeclampsia, pregnancy-induced hypertension, bleeding complications, preterm delivery, and spontaneous abortions (Harden et al., 2009). As such, women who are currently prescribed an antiepileptic medication (AEM), are encouraged to continue their regimen throughout their pregnancy. One such AEM is valproic acid (VPA), a short-chain fatty acid and histone deacetylase inhibitor that acts as a broad-spectrum anticonvulsant (Gurvich et al., 2005). Valproic acid has been approved to treat bipolar and other psychiatric disorders including schizophrenia and personality disorder. In fact, a study conducted from 2003-2007 found that more women have been prescribed AEMs for psychiatric disorders than those prescribed for epilepsy (Bobo et al., 2012). The number of women of child-bearing age who have been prescribed VPA has increased dramatically in recent decades (Lloyd, 2013; Atturu and Odelola, 2015). A correlation between AEMs and an increased risk (2-3x) for birth defects was first reported in 1968 (Meadow, 1968; Tomson and Battino, 2009; Palac and Meador, 2011). Valproic acid carries the greatest risk of all anti-epileptic medications for NTDs in offspring (Meador et al., 2008), with a dose-dependent risk for other defects as well, including impaired cognitive development (Bromley et al., 2010; Palac and Meador, 2011; Pennell et al., 2012). While the underlying mechanism wherein VPA induces
NTDs remains unknown, there is good evidence pointing to oxidative stress, a focus here with data presented later.

Genetics in Neural Tube Defects

The genetic complexity of the events leading to NT closure is vast. Women who have had an affected pregnancy are 2-5% (50-fold greater risk) more likely to have a subsequent NTD pregnancy (Copp et al., 2013). Several identified genetic factors contribute to NTDs in animals (Wilde et al., 2014), including mutations in ciliary signaling pathway, planar cell polarity pathway (PCP), cellular division and differentiation, NT patterning, cellular motility and cell adhesion components. Most of these genes have been identified in mouse mutants, while human genetic screening has been of interest in recent years. Additionally, epigenome analysis has revealed several mutations in acetylase, deacetylase, methylase, and demethylase proteins, as well as nucleosome remodeling enzymes as being linked to NTDs (Wilde et al., 2014). Developmental research has expanded the frontier of genetics to include the effects of gene-environment interactions.

Glutathione Redox Potential and Embryonic Development

Reactive oxygen species (ROS), such as hydrogen peroxide, nitrogen oxide, super oxide anion, and hydroxyl free radicals, are naturally occurring molecules with an unpaired electron, generated via the reduction of oxygen. The mitochondria are the primary source of ROS generation, as oxygen is reduced to generate ATP, accounting for up to 90% of all ROS production (Balaban et al., 2005). During development, cells are constantly undergoing growth, replication, migration, and differentiation, all of which require elevated levels of energy. This results in greater levels of ROS as a by-product of increased metabolism in the embryo.
Reactive oxygen species are often associated with deleterious outcomes such as lipid peroxidation or protein and DNA damage. However, in recent years evidence indicates that these low to moderate cellular levels of ROS may act as important signaling molecules that play a role in cellular activities, including cell cycle and proliferation, differentiation, migration, apoptosis, and antioxidant control (Allen and Tresini, 2000; Covarrubias et al., 2008; Albadri et al., 2019). For example, hydrogen peroxide may act as signaling molecule to activate angiogenesis (Gatenby and Gillies, 2004; Gao et al., 2007; Horak et al., 2010; Semenza and Prabhakar, 2012), cell growth and proliferation (Papapostolou et al., 2014; Wang et al., 2017), or cell survival (Groeger et al., 2009). If unregulated during development, these ROS can damage DNA, macromolecules, and cell membranes.

Exposure to developmental toxicants can induce the generation of high levels of ROS within a cell. Many environmental toxicants, such as xenoestrogens, pesticides, and heavy metals induce oxidative stress and are associated with greater risk for negative developmental outcomes. These toxicants have been associated with mitochondrial DNA damage/dysfunction, resulting in prenatal outcomes (Stohs et al., 2001; Ooe et al., 2005; Moon et al., 2012). Additionally, unhealthy practices by the mother may also lead to increased ROS generation and exposure to her unborn child during early development; smoking, alcohol consumption, and drug abuse have been linked to birth defects (Kovacic and Somanathan, 2006; Desrosiers et al., 2012). Unhealthy eating and metabolic disorders, such as maternal diabetes and obesity, are also associated with embryonic exposure to greater levels of ROS.

Antioxidant machinery within the cell can act to buffer the deleterious effects of high ROS generation and redox stress. Reducing couples, such as glutathione (GSH) and glutathione disulfide (GSSG), can be measured and used to calculate the redox potential ($E_h$), or capacity of
the couple to buffer ROS. Measuring the concentrations of oxidized and reduced forms of glutathione allows for a snapshot of the reducing power, or redox potential (Eₗ), of this antioxidant couple via the Nernst equation (Harris et al., 2013; Timme-Laragy et al., 2018). The GSH Eₗ is measured in negative mV; the more negative the Eₗ, the more reduced or capable GSH is to buffer redox stress, while the more positive the Eₗ, the more oxidized and incapable of buffering stress. As the redox state becomes more oxidized (less GSH), differentiation may occur. For example, in a more reduced state (high GSH concentration), cellular processes such as proliferation are favored. However, too much of an oxidizing shift in the GSH Eₗ can lead to apoptosis and necrosis, because GSH is no longer capable of buffering the redox stress. When measured over time, the GSH Eₗ reveals how constant or dynamic the redox state of the cells or tissue is during normal development, or in response to toxicant exposure. This tool has been employed in several developmental models, including zebrafish (Timme-Laragy et al., 2013), mouse (Gardiner and Reed, 1995), and rat (Jilek et al., 2015), to characterize normal and abnormal development.

Chicken Embryo Model

The chicken embryo has been used to study vertebrate embryo development since the 1600s (Stern, 2005). The chick is a relatively inexpensive model that allows for easy access to the embryo, without complicating maternal factors. During the early stages of development, the chicken embryo develops similarly to the human embryo. Manipulation of the chick embryo is easy via in or ex ovo techniques such as windowing or EC culture (Chapman et al., 2001). The chicken genome has been sequenced, allowing for the examination and discovery of key developmental genes.
The Hamburger-Hamilton (HH) stages are associated individual stages with hours/days of development post fertilization (3.5 h-21 days) and somite number (Hamburger and Hamilton, 1951). To examine the effects of toxicants on neural tube closure, eggs must be incubated to allow chick embryos to develop to HH Stage 8 (28-32 h), a stage described by 3-5 somite pairs having formed. At this point during development, the neural folds have risen and begin to fuse at the dorsal midbrain (Bellairs and Osmond, 2005). The anterior neural tube closes completely by HH stage 12 (16 somites), while the posterior neural tube closes between stages 13-14 (20-23 somites; (Schoenwolf, 1979). This closure is completed, in normal embryos, during the 24 h treatment window used in our experiments.

While working heavily with embryo collections and drug exposures, I was able to publish a methods book chapter in *Developmental Toxicology* (Stark and Ross, 2019), refining and introducing new procedures for utilizing the chick embryo as a model for toxicological studies. Methods described in later chapters include modifications and techniques that I have adapted for this work.
Study Design

The purpose of this research was to study the effects of three toxicants (ceramide, fumonisin and VPA) on embryo development using the chick embryo and cell culture models. Four distinct aims were proposed: 1) Assess the effects... of chicken embryos treated with ceramide, fumonisin or VPA, 2) evaluate changes in the oxidative state of embryonic tissue, 3) perform histological analysis of the neural tube, and 4) report differential gene expression profiles of chicken embryos treated with ceramide, fumonisin, or valproic acid.

Experimental Set Up

To determine the effects of C2-ceramide (C2), fumonisin B1 (FB1), and valproic acid (VPA) on undifferentiated and differentiated cells. This pluripotent Cells were cultured with various concentrations of C2, FB1, or VPA for up to 24 hours after which samples were collected to assess viability (MTT assay), changes in cellular redox state (glutathione redox potential analysis), and neuronal differentiation.

For embryo studies, chicken embryos (HH8) were incubated on 6-well agar plates with C2, FB1, or VPA for up to 24 hours. Embryos were collected and assessed for morphological and histological changes, staining included Pax3 (NTC transcription factor), PH3 (cellular proliferation marker), and LysoTracker (cell death marker). Glutathione (GSH) redox potential (Eh) was analyzed to assess changes in tissue redox state over a 24-hour period under each treatment. Embryos treated for 12 hours were collected for RNA sequencing analysis.
Specific Aims

- Characterize the effects of ceramide (C2), fumonisin (FB1), and valproic acid (VPA) on cellular viability, differentiation, and oxidative state.
- Evaluate morphological and histological changes in the developing neural tube of embryos treated with C2, FB1, or VPA.
- Determine whether changes in the redox state of embryonic tissue is altered under treatment conditions, and whether those changes are similar or different across treatments.
- Evaluate and compare gene expression profiles of embryos treated with C2, FB1, or VPA during neural tube closure.
Figure 1.3: Experimental Models and Treatment Timeline.

(A) P19 cells are cultured with C2, FB1, or VPA as undifferentiated cells, or initially treated with retinoic acid (RA) to induce neuronal differentiation prior to drug treatment. Cells were assessed for viability, reactive oxygen species (ROS) generation, glutathione redox potential (GSH E₉) analysis, and differentiation potential. (B) Chick embryos were collected from the egg at HH Stage 8 (3-5) somites and incubated with C2, FB1, or VPA for up to 24 hours. (C) Cell and embryo experimentation timeline.
CHAPTER 2: Embryonic Exposure to C2-ceramide Alters Embryonic Development and Increases the Frequency of Neural Tube Defects

Introduction

One of the major eukaryotic lipid groups, sphingolipids are associated with nearly every cellular pathway one might study. Ceramides are structural and functional membranous components located in the body at the center of sphingolipid metabolism (Castro et al., 2014; Bocheńska and Gabig-Cimińska, 2020). With a sphingolipid backbone linked to an acyl chain of 2-24+ carbons, various ceramide species influence cellular location and function throughout the body (Figure 2.1 A). Several metabolic and neurological diseases have been associated with altered expression of ceramides, including type two diabetes, obesity, Parkinson’s, Alzheimer’s, Gaucher’s, Crabbe’s, and Neimann-Pick’s disease (Haus et al., 2009; Brozinick et al., 2013; Mencarelli and Martinez-Martinez, 2013). In obese individuals, where global ceramide levels are elevated, bariatric surgery has shown to decrease overall weight and lower circulating plasma ceramide levels (Huang et al., 2011).

Ceramides are generated via three different pathways: de novo synthesis, the salvage pathway, and sphingomyelin hydrolysis (See Figure 2.1 B). De novo synthesis occurs within the outer leaflet of endoplasmic reticulum (ER) membrane (Michel et al., 1997) or within the mitochondria (Bionda et al., 2004), where serine and palmitoyl-CoA are condensed and reduced to produce sphinganine. Sphinganine is acetylated by ceramide synthase (CerS) to dihydroceramide, which is then converted to ceramide by ceramide dehydrogenase (Grosch et al., 2012).

Six human CerS enzymes have been identified, each displaying preference in generating certain ceramide species (Mullen et al., 2012; Park et al., 2014). CerS is activated in response to a host of stimuli, including heat stress, UV radiation, chemotherapy, apoptotic signals, stress...
response, apoptosis, and cell cycling (Rotolo et al., 2005; Siskind et al., 2010; Hoeferlin et al., 2013). Ceramide generation via different CerS are known to function distinctly within mammalian systems (Laviad et al., 2008; Park et al., 2014). For example, in insulin resistant metabolic disorders, C16-ceramides (CerS6) in the liver and C18-ceramides (CerS1) in skeletal muscle (Blachnio-Zabielska et al., 2016) contribute to insulin resistance, while CerS2 generated ceramides promote insulin sensitivity (Mahfouz et al., 2014; Raichur et al., 2014).

Ceramides influence cell death, differentiation, proliferation, migration, and cycle arrest. Other cellular and tissue fates, including autophagy, necrosis, mitophagy, cytoskeletal rearrangement, metabolism, and insulin signaling, are associated with ceramide mis-regulation.

Additionally, ceramide is created via hydrolysis and salvage pathways. Hydrolysis of sphingomyelin occurs in a similar fashion, generating ceramide and phosphorylcholine (Hannun, 1994). The enzymatic activity of SMase depends upon the pH, thus allowing for subcellular localization of sphingomyelin hydrolysis (Mencarelli and Martinez-Martinez, 2013). SMase concentration and location allow for variable ceramide populations and ratios within cells and the body. The salvage pathway utilizes the reverse functionality of the ceramidase (CDase) enzyme. Similar to SMase, CDase condensation of sphingosine and fatty acyl-CoA molecules generates new ceramides within the lysosomes (Bar et al., 2001), mitochondria (El Bawab et al., 2000), and nuclear membranes (Shiraishi et al., 2003).

Under normal physiological conditions, the most common ceramide species are C16:0 (palmitic) and C18:0 (stearic) fatty acids (Mencarelli and Martinez-Martinez, 2013). Long chain C18:0 is the most common ceramide within neural tissue. Under diabetic and obese conditions, serum C18:0, C20:0, and C24:1 basal concentrations are elevated, while C18:0 ceramides and
Figure 2.1: Ceramide Structure and Synthesis.

(A) Ceramides are members of the sphingolipid fatty acid family. Ceramide molecules contain an 18-carbon sphingoid backbone and an N-acetylated chain of 2-26 carbons (most common are ceramide species with N-acyl chains of 16-26 carbons). Example is a C16:0 molecule. (B) Ceramides are generated via three different pathways: de novo (endoplasmic reticulum), hydrolysis (plasma membrane), and salvage (lysosomes). Each pathway regulates ceramide localization, generating ceramides that perform different functions. Modification and packaging of ceramides into more complex glycosphingolipids occurs within the Golgi apparatus prior to cellular use and distribution.
total dihydroceramide concentrations are higher in T2D, correlating positively with insulin resistance (Haus et al., 2009; Lopez et al., 2013; Bergman et al., 2015).

Ceramides play an integral role in cellular processes such as stress response, cell cycling, cell death, and proliferation, serving as key regulators (Scarlatti et al., 2004). These bioactive lipids respond to stimuli and act as second messengers in systems associated with cellular differentiation, growth, and apoptosis, and thus purport a potential role during development (Jembrek et al., 2015). More specific to neurogenesis, lipid rafts of neuronal cells contain significantly higher levels of ceramides compared to levels observed in other somatic cells (Mencarelli and Martinez-Martinez, 2013). These rafts are important in neuronal cell adhesion (Povlsen and Ditlevsen, 2010) synaptic protein localization and nerve conduction (Hering et al., 2003), neurotransmitter receptor stabilization (Bruses et al., 2001), and axon guidance (Kamiguchi, 2006). Additionally, lipid rafts are involved in cell-cell interactions, protein binding, protein trafficking and signal transduction (Simons and Ikonen, 1997; Jin et al., 2011). Several neurodegenerative diseases have been associated with ceramide imbalance, including Alzheimer’s, Parkinson’s, Niemann-Pick’s, Gaucher’s, and Krabbe’s disease (Mencarelli and Martinez-Martinez, 2013). Autophagy is also regulated by ceramides through disrupting protein function and altering gene expression (Scarlatti et al., 2004). In studying the effects of long-chain highly hydrophobic fatty acids like C18:0, a short chain analog (C2 or C6) is often employed experimentally due to increased solubility and hydrophilic properties. While C2 and C6 exist within mammalian cells they are not the predominant species. Normally located in the brain and liver, the hydrophilic C2 is commonly used experimentally to mimic the longer ceramides in biological systems and is thought to be quickly modified to longer chain ceramides (Abe et al., 1996; Zheng et al., 1999; Mencarelli and Martinez-Martinez, 2013).
Ceramides have never been explored as a cause for NTDs, with only a few reports implicating components of the sphingolipid metabolism pathway (Sadler et al., 2002; Gelineau-van Waes et al., 2012). Environmental influences such as oxidative stress, have been well-documented in cases of NTDs, such as those associated with exposure with valproic acid (Tung and Winn, 2011), benzo-pyrenes (Lin et al., 2018), arsenic (Han et al., 2011), and in cases of folate deficiency (Lee et al., 2011). Interestingly, ceramides have been associated with oxidative stress (Quillet-Mary et al., 1997; Petrache et al., 2005; Kasumov et al., 2015). Linking elevated ceramides and an increased risk for NTDs in obesity and diabetes, we hypothesized that altered ceramide profiles under conditions of maternal obesity induce oxidative stress in embryonic tissue, resulting in an increased risk for neural tube or other defects. This study used a combination of P19 cells and chick embryos to test for in vitro and in vivo effects and potential mechanisms of ceramide exposure on neural tube closure.

Methods

Cell Culture

P19 mouse embryonal carcinoma cells were obtained from American Type Culture Collections (ATCC) and cultured in alpha-modified minimum essential medium (Thermo Fisher) supplemented with 7.5% (v/v) bovine calf serum (ATCC), 2.5% (v/v) fetal bovine serum (Genesee), 100 units/mL penicillin (Genesee), 100 μg/mL streptomycin (Genesee), in a humidified incubator gassed at 5% CO2 and heated to 37 °C.

Neuronal differentiation was carried out as described per Laplante et al. (2004). In brief, cells were seeded at a density of 1 x 10⁶ cells per 60 mm bacteria grade petri dish, treated with 1 μM retinoic acid (Sigma-Aldrich), and cultured as aggregates from day 0 to day 4 (D0-D4). On
D4, aggregates were removed and centrifuged at 1000 rpm for 5 min at room temperature. The pellets were dissociated in 0.05 % trypsin (Genesee) supplemented with 10 μL/mL of 1,000 U DNase 1 (Thermo Fisher) and incubated in a water bath at 37 °C for 5 min with gentle rocking. Single cells were seeded at a density of 40,000 cells/cm² on gelatin-coated tissue culture dishes in Neurobasal medium (Thermo Fisher) supplemented with 0.5 mM L-glutamine (Genesee), 2% (v/v) B27 supplement (Thermo Fisher), 100 units/mL penicillin, and 100 μg/mL streptomycin until treatment on D7.

**Embryo Culture**

The EC culture method has been employed in our lab to observe chick embryo development under tightly controlled conditions, including in the presence of developmental pathway inhibitors (Voelkel et al., 2014). Because the chemical inhibitors are added to the agar bed that underlies the embryo in EC culture, higher concentrations are used than in cell culture experiments.

Fertilized chicken eggs were obtained from local vendors and stored at 12-17°C for up to two weeks. Eggs were incubated at 38.5°C for 28-32 h to Hamburger & Hamilton (HH) stage 8 (3-5 somite stage). Embryos were collected using early chick (EC) culture method described Chapman *et al.* (2001). Briefly, for each embryo, an egg was cracked into a 100-mm petri dish with the embryo floating atop the yolk. Thick albumin was retracted from the top of the yolk near the embryo and a filter paper disc (1” disc with a ½” hole in the center) was placed on the yolk with the embryo in the center. Each embryo was collected by cutting the vitelline membrane completely around the disc and carefully lifting the disc with the adhered vitelline membrane and suspended embryo from the yolk. A second filter paper disc was placed on the ventral side to create a sandwich. Embryo discs were placed in a 100-mm petri dish with fresh 1x PBS, and
subsequently plated on DMSO control (0.5% DMSO) or C2-ceramide (C2; 50-100 µM) treated six-well agar plates (described under Ceramide Dose Curve). Embryos were incubated on the plates for a specified time before being removed into fresh 1x PBS for imaging.

**MTT Assay**

P19 cells were seeded at a density of 1 x 10^5 cells/well in a 96-well plate and treated with varying concentrations of C2 (6.25-800 µM) for 24 h. Following exposure, cell viability was measured using a 3-[4,5- dimethylthiazol-2- yl]-2,5 diphenyltetrazolium bromide (MTT) assay, which is based on the ability of cells to cleave MTT to an insoluble violet formazan product by the activity of mitochondrial dehydrogenases. At the end of the treatment period, 0.5 mg/mL MTT (Sigma-Aldrich) was added to the medium and incubated for 3 h at 37°C. The formazan dye was dissolved by adding 100 µL DMSO to each well, and the absorbance was measured with an automatic microplate reader at 590 nm.

**Dose Curve Analysis**

Long chain ceramides (C16-C24), while more physiologically relevant are hydrophobic and difficult to study. Therefore, the hydrophilic short chain ceramide C2 is used as an experimental analog to study the effects of ceramide-mediated processes. C2 concentrations of 0 µM, 50 µM and 100 µM were tested to determine frequency of NTDs and development in chicken embryos. Chicken embryos were incubated to HH8 and collected onto C2 treated plates of 0 µM, 50 µM and 100 µM. Embryos were incubated for 24 h, after which somite count and morphological analysis were compared between groups. Whole and sectioned embryos were evaluated to determine open and closed NTDs. Six-well plate preparation for chicken embryo culture was as follows:
Ceramide Plate

A C2 (Cayman Chemical) stock solution of 20.91 mM (1 mg/140 μL DMSO) was diluted in 2 mL 1%BSA/PBS with PenStrep (1:1000) to 25, 50, or 100 μM. 6 mL thin albumin was added, followed by 6 mL agar (0.18 g BactoAgar in 6 mL saline) to produce 14 mL homogenized drug solution. 2 mL of the solution was added to each well of a 6 well plate and refrigerated to solidify.

Control Plate

70 μL Dimethyl Sulfoxide (DMSO; Millipore) was added to 2 mL 1%BSA/PBS with PenStrep (1:1000). 6 mL thin albumin was added, followed by 6 mL Agar (0.18 g BactoAgar in saline) to produce 14 mL homogenized drug solution. 2 mL of the solution was added to each well of a 6 well plate and refrigerated to solidify.

Morphological Analysis

Embryos were incubated sequentially at 37 ºC for 2 hours in 5% and 15% sucrose in 1x PBS before embedded in gelatin for cryosectioning. Twelve μm sections were mounted on Superfrost Plus glass slides and gelatin was removed in PBS for ten minutes at 37 ºC. Embryo sections were incubated with 1:500 dilution of laminin primary antibody (3h11; Developmental Studies Hybridoma Bank) or 1:250 Pax3 (Developmental Hybridoma Bank) in 0.1% BSA/PBS (bovine serum albumen, with 0.1% Tween-20) at 4 ºC overnight. The primary antibody was rinsed twice in PBS for ten minutes at room temperature before being covered with 1:1000 dilution of Alexa-488-conjugated goat anti-mouse IgG2a (Invitrogen), Alexa-546-conjugated anti-rabbit IgG (Invitrogen), or Alexa-633-conjugated goat anti-mouse IgG1 (Invitrogen) in BSA/PBS buffer. Tissue sections were incubated for one hour at room temperature. Slides were
then rinsed in PBS for ten minutes. DAPI (Sigma) was used to visualize nuclei with 1:10,000 dilution. Antibody staining was visualized and imaged using an Olympus BX-61 fluorescent microscope and CellSense software.

Statistics

Data was analyzed using Microsoft Excel 2016 and Prism 7 software. A confidence interval of 95% ($\alpha=0.05$) was used as threshold for statistical significance. Data are presented as mean ± SEM, with sample size ($n$) specified in the corresponding figure.

*P19 Cells.* Statistical significance was measured with Two-Way ANOVA and Dunnett’s multiple comparison tests (MTT) or Tukey’s multiple comparison tests (GSH and DCF).

*Chick Embryos.* Statistical analysis was conducted with Two-Way ANOVA and Bonferroni (GSH, PH3, and LT) multiple comparison tests, Paired T-Test (DCF), Unpaired T-test (somite number) or Kruskal-Wallace and Welch’s t-tests (dose curve, NTD type).

Results

To understand the mechanism leading to embryo defects, it was important to evaluate the overall cellular effects of C2-ceramide (C2) in a tightly controlled in vitro system. We first explored the effects of C2 on an immortalized cell line (P19 cells) that could be differentiated into neuronal precursors, as the neural tube is the structure to give rise to the brain and spinal cord. We discovered that shifts in redox potential occurred, and there was some evidence of oxidative stress in the absence of apoptosis or necrosis. When comparing cell culture data with observations in embryo tissue, some similarities, but also important differences, were observed.
Cell Viability is Unchanged, but ROS Accumulates in C2-Treated P19 Cells

To more comprehensively assess the effects of C2 on early neurulation, assays were done in cell culture, thereby informing future experiments in embryos. P19 cells were selected due to their similarity to neuronal precursor cells (Kim et al., 2001), such as might be found in the early neural tube. Moreover, P19 cells have been used previously as a model of neurogenesis and neurotoxicity (Pashkovskaia et al., 2018) and diabetic embryopathy (Chang et al., 2003), and thus provide a reliable, effective means to evaluate the effects of C2 in vitro.

With any toxicant, cellular damage leading to necrosis or apoptosis is an important consideration. Undifferentiated P19 cell viability was assessed using several concentrations of C2, ranging from 6.25-200 µM (n=4 at each dose tested). Cell viability, as assessed by culturing undifferentiated P19 cells with 100 µM DCF-DA and then treating with different concentrations of C2 (6.25-800 µM) and measuring changes in fluorescent intensity, was not affected except at 200 µM (Figure 2.2 A), where it decreased significantly (p<0.0001). From this, we concluded that C2 exposure below 200 µM was not cytotoxic to undifferentiated P19 cells, thereby informing planned experiments in embryos.

Because ceramide is known to induce oxidative stress, ROS generation was measured in P19 cells exposed to non-cytotoxic concentrations of C2 (25-100 µM) using the DCF assay. ROS generation increased in undifferentiated P19 cells in a dose-dependent manner (Figure 2.2 B), as shown by a significant increase in the percent change of fluorescence being seen at 75 µM (p=0.0413) and 100 µM (p=0.015), showing that ROS is generated in absence of cell death. We therefore hypothesized that ROS generation would be seen in undifferentiated neuroepithelial cells in embryos.
Figure 2.2: P19 Cell Viability and Reactive Oxygen Species Generation. Undifferentiated P19 neural precursor cell viability was assessed using an MTT Assay to determine the toxicity of C2 on living cells (A). P19 cells were exposed to 6.25-200 µM concentrations for 24 h (n=4). Cell viability dropped significantly only at 200 µM C2 (p<0.001). Reactive oxygen species (ROS) generation increased significantly in C2 treated undifferentiated P19 cells at 75 µM (p=0.0413) and 100 µM (p=0.015), as indicated by the change in percent of DCF fluorescence compared to control (n=4). Asterisks denote significance between 100 µM and other treatments (* = p≤0.05, ** = p≤0.01, *** = p≤0.001, **** = p≤0.0001).
Glutathione Analysis of Differentiated and Undifferentiated P19 Cells Reveals Unique Cellular Redox Shifts After C2 Treatment

With results showing good cellular viability, but ROS accumulation after C2 exposure between 75-100 µM concentrations, we assessed shifts in the cellular redox state of P19 cells as measured by glutathione redox couple HPLC analysis. Undifferentiated P19 cells were exposed to C2 (10-100 µM) over 48 h to determine the effect on cellular GSH $E_h$ (Figure 2.3 A). For most treatment concentrations, no differences were observed at any time point between experimental and control samples, including at 100 µM C2.

Interestingly, no differences occurred in total concentrations of GSH and GSSG in the undifferentiated C2-treated P19 samples compared to controls (Figure 2.3 B-C), indicating C2 treatment does not alter GSH concentrations or redox potential.

It is known that cells and tissues can exhibit dramatically different redox profiles depending on their differentiation state (Hansen and Harris, 2015). To evaluate whether C2 exposure affected the GSH $E_h$ in a model of differentiated, neuronal cells, P19 cells were differentiated into neurons, and then treated with varying concentrations of C2 (10-100 µM), with measurements taken at 4, 8, 12, 24, 36, and 48 h (Figure 2.3 D). When comparing treatments to controls, GSH $E_h$ was more oxidized only in differentiated cells treated with 100 µM C2, beginning at 24 h and continued through 48 h (p<0.0001).

At 24 h, GSH concentrations were significantly decreased in differentiated cells treated with 100 µM C2 compared to controls (p=0.0003; Figure 2.3 E). Lower GSH concentrations were observed in 50 µM C2-treated samples when compared to control at 24 h (p=0.0455) and 36 h (p=0.0008). No differences were seen at any time point with 10 µM C2. Differentiated P19
Figure 2.3: Glutathione Analysis of C2-Ceramide Treated Differentiated and Undifferentiated P19 Cells over 48 hour Period.

(A-C) Undifferentiated P19 cells were treated with 10, 50, or 100 µM C2 for 4, 8, 12, 24, 36 or 48 h and collected for GSH redox potential ($E_h$) analysis. No significant difference was observed between treatments and control. (A). Glutathione and GSSG concentrations did not vary between treatments (B, C). D-F. Differentiated P19 cells were similarly incubated and collected for GSH $E_h$ analysis. 100 µM C2 treatment induced a significant oxidizing shift at 24, 36 and 48 h in the GSH $E_h$ when compared to controls (p<0.0001; D). A comparison of GSH concentrations show significantly lower concentrations in 100 µM C2 when compared to control 24 h (p=0.0001), 36 h (p=0.0001), and 48 h (p=0.0004; E). 50 µM C2-treated samples showed lower GSH levels when compared to control at 24 h (p=0.0455) and 36 h (p=0.0008). GSSG concentrations differed between control and 100 µM C2 at 36 h (p=0.009) and 48 h (p=0.0368; F). Asterisks denote significance between 100 µM and control (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001). # = significance between 50 µM C2 and control.
GSSG concentrations were lower with 100 µM C2 treatment compared to control at 36 h (p=0.009), and at 48 h (p=0.0368; Figure 2.3 F).

Cellular redox states have been shown to become increasingly oxidized as cells become increasingly differentiated. Here, P19 cell redox states recapture that feature and also shift to a more oxidizing redox state as neurogenesis occurs. As such, susceptibility to C2 treatments between undifferentiated and differentiated P19 cells may be a consequence of inherent redox shifts in the GSH $E_h$ during differentiation to a more oxidizing state, providing rationale as to why more reducing, undifferentiated cells are more resistant to C2-induced oxidation. While the preliminary P19 cell work informed future direction, experimentation in whole embryos depicts more accurately the effects of C2 in a system that contains numerous cells types, including neuronal precursors, in a three-dimensional structure.

Cellular Proliferation is Decreased, While Cell Death and Embryo Viability are Unchanged Under C2 Treatment

Using the P19 cell culture data above to inform embryo experimental design, we first looked at cell proliferation and death, and ROS accumulation. Phospho-histone 3 (PH3) is a marker for cellular mitosis and is thus used to visualize proliferating cells. Chick embryos (HH8) were incubated on C2- or DMSO-treated agar plates for 24 hours. Embryos were then sectioned and stained with PH3 antibody. Sections were grouped into four regions of interest: Head, Otic, Upper Spinal Cord (USC), and Lower Spinal Cord (LSC). Total count and count within the neural tube of PH3+ cells were measured for each section. As shown in Table 2.1 and Figure 2.4, most regions showed a statistically significant reduction in PH3+ cell after C2 treatment, even though high variability was observed across sections in both C2-treated and
Table 2.1: Comparison of the Cellular Proliferation Globally and Within the Neural Tubes of C2-Treated and Control Embryos.

Tissue sections were analyzed for C2- and DMSO-treated embryos to obtain PH3+ (marker for M phase) cell counts. Sections were grouped by region based on location along the A-P axis in four groups: Head, Otic, Upper Spinal Cord (USC), and Lower Spinal Cord (LSC). Due to high variability, an average PH3+ cell count is reported for all sections and an average within the neural tube (NT). A sample size of 4-5 embryos was analyzed per group. Head: PH3+ cell counts were significantly different for both total and NT averages (p<0.0001). Otic: No significance was observed between treated (n=5) and control (n=5) total PH3+ count (p=0.4622), however, significance was observed between average PH3+ cell counts within the neural tube (p=0.0181). USC: PH3+ cell counts varied significantly between DMSO (n=5) and C2-treated (n=5) embryos both in total average count, 46.06 (p=0.0006), and in NT average count, 13.04 (p<0.0001). LSC: Significance was observed between average cell counts in the lower region of C2 (n=5) and DMSO (n=4) treated embryos in total PH3+ cell count (p=0.034), however no significant difference was seen in the average number of proliferating cells within the NT (p=0.8957).

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<td>10.64 (±1.047)</td>
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<td>46.06 (±2.751)</td>
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<tr>
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<td>7.967 (±0.5459)</td>
<td>5</td>
<td>54</td>
<td>34.37 (±2.172)</td>
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Figure 2.4: PH3 Cellular Proliferation Analysis of C2-Ceramide Treatment. C2- and DMSO-treated embryonic tissue sections were analyzed and PH3+ (marker for M phase) cell counts were collected to measure cellular proliferation, both globally and within the neural tube. Sections were grouped by region based on location along the A-P axis in four groups: Head, Otic, Upper Spinal Cord (USC), and Lower Spinal Cord (LSC). Global cellular proliferation was significantly reduced in embryonic sections within the Head (<0.0001), USC (p=0.0006), and LSC (p=0.034) regions. Within the neural tube, fewer proliferating cells were counted in the Head (p<0.0001), Otic (p=0.018), and USC (p<0.0001) regions.
control embryos. The *Otic* region was not significantly different when counting cells in the entire section (p=0.4622), and the *LSC* region was not significantly different when counting PH3+ cells in the neural tube (p=0.8957). Overall, we conclude that C2 treatment resulted in a global reduction in cell proliferation.

To assess early apoptosis, embryos were cultured in the presence of DMSO (control; n=5) or 100 µM C2 (n=6) for 24 h. LysoTracker Red (LT) punctae, a readout of acidic lysosomes and a marker of apoptosis, were quantified in histological sections (Figure 2.5 A-B). Results revealed that when comparing control and C2-treated embryos, no significant difference was found in the total number of LT-positive acidic lysosomes (p=0.8106), or in the number of LT-positive acidic lysosomes found just within the neural tube (Figure 2.5 C). Cranial vs. caudal sections and open vs. closed neural tubes also showed no difference (data not shown).

**ROS Generation is Unaffected, but Glutathione Redox States are Altered in C2-Treated Embryos**

To determine whether there was a difference in ROS generation in embryos, we employed a whole-mount DCF assay. Measurements were specifically taken from the developing cranial neural folds and the area adjacent to the somites (Figure 2.6 A-B). A comparison of the relative DCF fluorescence in the neural folds of embryos incubated in 100 µM C2 indicates that overall ROS generation increases an average of 45.06% in C2-treated embryos compared to 41.6% in controls in a 16-minute time course (Figure 2.6 C). While the neural folds of the C2-treated embryos (Figure 2.6 B) appeared brighter than those of the control embryos (Figure 2.6 A), no statistical significance was observed.

Early stage chick embryos (3-5 somite stage) were exposed to 100 µM C2-ceramide for several time points from one to 24 h and evaluated for changes to GSH E°h. Exposure to C2
Figure 2.5: Embryonic Apoptosis is Unaffected by C2-Ceramide Treatment. (A-D) LysoTracker Red Lysosomal Stain. Analysis of control (n=5) and C2 treated embryos (n=6) revealed there is no difference in cell death activity throughout the embryo or within the neural tube (A-B). Quantification revealed no statistical difference in the total number of acidic lysosomal punctae or number of punctae within the neural tube (C). No difference was observed between cranial or caudal sections (data not shown).
Figure 2.6: ROS Generation Unchanged After Embryonic C2-Ceramide Treatment. Analysis of reactive oxygen species (ROS) generation was measured in HH8 chick embryos treated with DMSO (A) or C2 (B) using a DCF assay. Percent change in relative fluorescence over a 16 min period showed no statistical difference in ROS generation between control and C2 embryos (p=0.2104; C).
quickly led to an oxidizing shift in embryonic GSH redox state at 1 h (p<0.0001), 2 h (p=0.0096), 4 h (p<0.0001), and 8 h (p=0.0006) when compared to control embryos (Figure 2.7 A).

Embryonic GSH concentrations were significantly lower compared to controls at 1, 2, 4, and 8 h (p<0.0001) after incubation (Figure 2.7 B). Interestingly, GSSG concentrations did not vary at any time point (Figure 2.7 C). Total GSH levels (\([\text{reduced GSH} + 2\text{oxidized GSSG}]\)) were significantly reduced between 1-8 h after incubation (p<0.0001; Figure 2.7 D). Albeit the timing of GSH redox changes is different in cells vs. embryos, similarities in C2-induced redox changes between models occur, where decreased GSH drives changes to the cellular GSH Eh, not an increase in GSSG.

**Pax3 Expression is Unchanged in C2-Treated Embryos**

Pax3 is a transcription factor known to regulate several key developmental genes, including many involved in neural tube closure (Mayanil et al., 2001; Sanders et al., 2014). Under hyperglycemic conditions, such as those found in maternal diabetes, oxidative stress has been shown to disrupt Pax3 expression (Li et al., 2005; Loeken, 2005). While C2 treatment did not result in ROS generation as measured by DCF, a clear shift in the GSH Eh was observed. Therefore, we examined whether C2 treatment altered Pax3 expression in exposed embryos (n=5) compared to control embryos (n=5). No difference was observed in Pax3 expression between DMSO control and C2-treated embryos within the ectoderm, neural crest cells, dorsal neural tube, or somites (see Figure 2.8). Additionally, no Pax3 expression was observed in control or treated embryos in regions where NTDs were observed in the caudal end where segmental plate mesoderm has not yet formed somites (see Figure 2.8 “Caudal” and Figure 2.10 C).
Figure 2.7: Glutathione Analysis of C2-Ceramide Treated Chick Embryos over 24 h Period. HH8 chick embryos were incubated on agar plates containing 100 µM C2 for 1, 2, 4, 8, 12, or 24 h, and collected for GSH analysis. A. Glutathione (GSH) redox potential (Eh) analysis revealed exposure to C2 resulted in an oxidizing shift in the Eh of the tissue that was significantly different when compared to control at 1 h (p<0.0001), 2 h (p=0.0096), 4 h (p<0.0001), and 8 h (p=0.0006). By 12 h, the state of the tissue recovered to normal conditions. B. Reduced GSH concentrations were significantly lower at 1, 2, 4, and 8 h after incubation (p<0.0001) when compared to control. C. GSSG concentrations were not different from control at any point over the 24 h time course. D. Total GSH concentrations (total GSH=[reduced GSH] + 2[oxidized GSH]) were significantly reduced in C2 treated embryos at 1 h, 2 h, 4 h, and 8 h when compared to control (p<0.0001). Asterisks denote significance between 100 µM and other treatments (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
Figure 2.8: Pax3 Expression Profiles in Response to C2-Ceramide Treatment. Pax3 expression was evaluated in regions along the A-P axis of C2-ceramide and DMSO treated embryos (n=5). Embryonic sections were grouped according to their location within the embryo into five groups: Head, Otic, Upper Spinal Cord (USC), Lower Spinal Cord (LSC), and Caudal. Pax3 expression was present in the ectoderm and migrating neural crest (Head), dorsal neural tube (USC, LSC), and somatic tissue (USC, LSC) regardless of neural tube closure. Note: Cranial NTDs were present in 2/5 of the C2-treated embryos (Otic and Caudal sections show defects).
C2-Ceramide Causes an Increased Incidence of NTDs in Chick Embryos

Using the EC culture method, embryonic morphology was characterized in whole-mount, with histological sections used to confirm gross observations of neural tube closure. Embryos were cultured for 24 h with DMSO, and 50 or 100 µM C2, with C2-treatment increasing the frequency of failed neural tube closure along the length of the A-P axis (Figure 2.9 C-D). Neural tube defects occurred at the following rates: 12% in control embryos; 14% in 50 µM embryos; and 45% in 100 µM embryos (Figure 2.9 E). While 50 µM C2 NTD rates were not statistically different from controls, NTD prevalence of embryos treated with 100 µM was statistically higher than that of control (p=0.0159). The seemingly high rate of NTDs in controls (12%) may be due to the methodology used, where embryos are not cultured in ovo. However, the advantages of EC culture allows for precise delivery and dosage of potential toxicants over time. The significant increase in NTD occurrence after C2 exposure (45%), along with the other embryo data presented shows NTDs occurring at a very high rate in the absence of detectable cytotoxicity in embryos.

Somite formation was measured after 24 h in 100 µM C2-treated embryos (n=31) to determine differences in overall growth and rate of development over time and compared to control embryos (n=23). Initial somite number was recorded for each embryo prior to treatment, with final somite number recorded upon embryo collection, and the difference was calculated (Figure 2.9 F). No differences were observed in starting somite number mean (Control: 4.8±0.13 vs. C2: 4.5±0.13), ending somite number mean (Control: 20.5±0.40 vs. C2: 19.3±0.67) or difference in somites added over time (Control: 15.7±0.42 vs. C2:14.9±2.9). While the SD was larger for C2-treated embryos, there was no correlation between starting somite number and somites added over time. Therefore, no statistical difference was observed in embryo growth.
Figure 2.9: Ceramide Dose Curve in Chick Embryos.

A-D. Observed Defects. Affected embryos exposed to 100 µM C2 developed cranial and caudal defects like what is observed in human embryos with neural tube defects. Normal embryonic growth was observed in control embryos (A). Cranial defects included minor as well as more severe defects, including failed neural fold bending (B). Caudal defects like that shown in C were commonly observed in affected embryos (somite formation was unaffected at the level of the open folds). Open neural folds and failed vesicle formation, with a cruciform defect above the otic cups, was also observed in some ceramide embryos (D). 

E. Ceramide Dose Curve. Chick embryos were incubated on control (0.5% DMSO) or C2 containing (50 µM or 100 µM) agar plates for 24 h. Control embryos experienced a neural tube defect (NTD) frequency of 13% (n=24). Exposure to 50 µM ceramide resulted in a 14% NTD frequency (n=21), while 100 µM C2 increased the frequency to 45% (n=44). 100 µM C2-induced NTD rate was statistically significant when compared to control (p=0.0021) and 50 µM (p=0.0061). 

F. Total Increase in Somite Pair Formation. No difference was observed in somite pair formation of treated embryos when compared to control. C2 treated embryos developed an average of 14.8±2.9 somites over 24 h, while control embryos developed 15.7±0.4 somites (p=0.2749). Asterisks denote significance between 100 µM and other treatments (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
No differences were observed in embryo survival, as measured by viable heartbeat after 24 h incubation, and gross anatomical differences such as overall length or distorted features were not obvious, although absolute measurements in embryo length were not recorded.

To confirm open or closed status of the neural tube, histological analysis was performed on treated and control embryos. Neural tube closure was evaluated, and defects were classified as cranial only (defects from the anterior neuropore to the 10th somite; Figure 2.10 A-B), caudal only (defects from the 11th somite to the posterior neuropore; Figure 2.10 C), or both cranial+caudal (defects in both the cranial and caudal regions). DMSO control sections with closed neural tubes are shown for comparison (Figure 2.10 A-C). A comparison of the number of embryos with cranial, caudal, or cranial+caudal defects (Figure 2.10 D) revealed a significantly greater number of embryos with cranial defects (n=10) compared to embryos with both cranial+caudal defects (n=4; p=0.0052). There was no significance between the number of embryos with cranial defects vs. caudal defects (n=6; p=0.0785) or embryos with caudal vs. embryos with cranial+caudal defects (p=0.512).

Discussion

The data presented here show for the first time that embryonic exposure to C2-ceramide (C2) resulted in an increased incidence of NTDs in early vertebrate embryos. As with many environmental factors that influence development, the mechanism is elusive, but data presented in this initial report supports redox dysregulation through the GSH E$_h$ as a potential effector.

By initially using P19 mouse embryonic carcinoma cells as an in vitro model to evaluate the effects of C2 exposure on neuronal precursor cells, we were able to establish several important pieces of information. For example, proper C2 concentrations for embryo exposure were determined where concentrations below 200 µM were non-cytotoxic, and the establishment
Figure 2.10: C2-Ceramide Induced Neural Tube Defects. (A-D) Cranial, caudal, and both cranial + caudal neural tube defects (NTDs) were visible in C2 ceramide (C2)-treated embryos. Brightfield and fluorescent images of cross sections show cranial NTDs in C2-treated embryos in the rostral hindbrain (A), the caudal hindbrain (B), and the caudal region of the embryo (C), compared to closed neural tube sections of control (DMSO-treated) embryos. Laminin and DAPI overlays for each section shows a continuous basement membrane in DMSO-treated embryos and a clear opening in the neural tube of C2-treated embryos. D. NTD Type Comparison. A comparison of the number of NTDs observed in C2 treated embryos. The number of embryos with cranial defects was significantly different from the number of embryos with cranial+caudal defects (p=0.0052). Asterisks denote significance between 100 µM and other treatments (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
of C2-induced ROS generation at concentrations of $\geq 75$ µM (Figure 2.2 A-B). From this preliminary data, we used 50 and 100 µM C2 concentrations in subsequent embryo experiments. The cell culture data also revealed interesting differences between differentiated and undifferentiated P19 cells. This observation was important, since the tissue of a developing embryo is rich with various cell types, including undifferentiated, differentiating, and differentiated cells. Interestingly, past studies showed that some cell types, including human neuroblastoma, PC12, and rat hippocampal cells, are sensitive at lower concentrations (France-Lanord et al., 1997; Darios et al., 2003; Prasad et al., 2008; Czubowicz and Strosznajder, 2014), suggesting some cell types are more vulnerable to the oxidizing effects of C2 than others. Here, differences were observed when comparing the effects of C2 on cellular redox state of undifferentiated vs. differentiated P19 embryonic carcinoma cells. Glutathione redox potential (GSH $E_h$) measurements revealed no difference between C2-treated and control treated samples in undifferentiated cells (Figure 2.3 A), while in differentiated cells, GSH $E_h$ was altered, becoming significantly more oxidized (Figure 2.3 D). These findings are consistent with previous research that shows similar trends of differentiated cells having more oxidizing GSH $E_h$ than their undifferentiated counterparts during adipogenesis and osteogenesis (Imhoff & Hansen, 2011). The initial redox state of differentiated P19 cells is more oxidized than that of undifferentiated cells. Therefore, C2 exposure results in a greater oxidizing shift (toward a state that would lead to apoptosis or necrosis) only in differentiated P19 cells. Clearly, the differences require more in-depth study to better understand these outcomes, but these initial results suggest cellular susceptibility based on the state of differentiation.
Embryonic Defect Data

In chicken embryos, the neural folds begin to fuse in the midbrain by developmental stage HH8 (3-5 somites; (Bellairs and Osmond, 2005). Closure of the anterior neuropore is complete by stage 12 (16 somites), and the posterior neuropore closes between stages 13-14 (20-23 somites; (Schoenwolf, 1979). Therefore, to analyze the effects of C2 exposure on the redox state of the tissue during the period of neural tube closure (26-53 h after fertilization), embryos were cultured on six well agar plates with 100 µM C2 and compared to control embryos. Neural tube defects were seen in 45% of C2 treated embryos, a frequency 3x that of control embryos (Figure 2.9 A-D). To understand potential mechanisms, we examined cell proliferation and death, ROS generation, pax3 expression, and redox potential.

Fewer PH3+ cells were observed in embryos after C2 treatment, indicating a reduction in cell proliferation. This change in cell proliferation is minor enough, or has late enough onset, so as to not create an obvious difference in embryo size, since this was not a consistent observation through the many experiments performed over time. Two potential hypotheses for this reduced proliferation are 1) an overall reduction in cellular metabolism due to C2 treatment impacting mitochondrial function, and 2) ciliary disruption due to C2 treatment disrupts cellular functionality and division. Ceramides are implicated in reducing the mitochondrial respiration of skeletal muscle cells (Hodson et al., 2015; Taylor et al., 2017), and several lines of study have indicated a potential role of primary cilium in the development of NTDs (Murdoch and Copp, 2010; Vogel et al., 2012; Toriyama et al., 2017; Juriloff and Harris, 2018). Ceramides are reported to regulate the generation and elongation of primary cilium (Wang et al., 2009; He et al., 2014). Future studies will examine cellular metabolism in the early embryo and explore the
role of ceramides in regulating primary cilia during neural tube closure under high C2 conditions.

Prior work by several labs identified abnormal cell death as causative for NTDs (Shimabukuro et al., 1998; Kamocki et al., 2013; Chang et al., 2015; Jazvinscak Jembrek et al., 2015). Therefore, it was important to determine whether exposure to C2 during development increased apoptotic activity within the neural tube. We employed the LysoTracker Red stain as an early marker to visualize, count, and measure activated lysosomes. Previous studies showed LysoTracker Red analyses as a comparable assay for areas of increasing apoptotic activity when compared to TUNEL staining, perhaps marking apoptotic cells earlier (Fogel et al., 2012). We observed no difference in the number of lysosomal punctae within the neural tube or throughout the embryo (Figure 2.5 A-C), likely ruling out increased cell death as a potential cause for C2-induced NTDs. Additionally, no increase in ROS generation within the neural folds was observed as measured by DCF analysis, a noted difference when comparing P19 cells to embryos. However, GSH $E_h$ was significantly altered in embryos during the first 8 h after exposure, with tissue GSH redox state recovering by 12 h (Figure 2.6). Therefore, an early, acute change in cellular redox state was observed. Furthermore, since GSH $E_h$ can be modified through processes other than ROS production, one possible explanation is a period of redox imbalance occurring in the first 8 h of exposure perturbs a developmental signaling pathway important for neural tube closure, since ROS-mediated changes to redox state are not the only means by which redox states are modified. Alterations to enzyme function, ATP availability and metabolism may be manifested as changes to intracellular redox state and do not necessarily occur as a consequence of ROS generation. This reasoning introduces and rationalizes many different pathological and developmental outcomes where redox couple “circuits” are modified.
to alter cellular function and adaptation (Jones, 2008). Since redox states and related circuits are the primary regulators of redox signaling, either conserved or pathologic (Schafer and Buettner, 2001), changes to redox state is of primary interest here. The observed shift in redox state in C2-exposed embryonic tissue in the absence of ROS or cell death points to changes in cellular functionality, signaling, processing, proliferation, and/or differentiation as potential factors causing neural tube defects.

Finally, we noted a greater number of cranial defects in C2-treated embryos (Figure 2.10 D). Caudal defects were observed, but at a lower, not significant, frequency. The frequency of cranial neural tube defects suggests anencephaly as a primary outcome (Carmichael et al., 2010), and could be due to susceptible developmental periods, or due to different mechanisms of tube closure cranially vs. caudally. Importantly, the increase in NTDs occurred with minimal impact on other embryonic tissues, since embryo survivability, heart formation and function, growth rate as measured by somite number (Figure 2.9 F), and other gross anatomical features, did not differ between DMSO and C2-treated chick embryos.

To summarize, we observed C2-induced shifts in GSH E_h (a measure of redox state) in treated embryos without a corresponding increase in apoptotic activity or ROS accumulation. Notably, cell proliferation was lower. The direct role of ceramides in disrupting proper neural tube closure is the focus of future planned experiments, which will more fully determine the mechanism by assessing changes in tissue lipidomics, gene expression, and cellular function.
CHAPTER 3: Embryonic Exposure to Ceramide, Fumonisin B1, and Valproic Acid During Neural Tube Closure: A Toxicological Comparison in the Chick Embryo

Introduction

More than 300,000 infants are born with neural tube defects (NTDs) worldwide each year. The neural tube (NT) is the first embryonic structure to form, during week 4 of human development, later becoming the brain and spinal cord of the developing embryo. The NT begins closing at the base of the hindbrain, extending anteriorly toward the face and posteriorly toward the back. Anterior defects most commonly result in anencephaly, a loss of skull bones and brain matter, while posterior defects result in various forms of spina bifida, an opening or protrusion of the spinal cord tissue in the lower back affecting spinal fluid regulation, bowel control, and lower limb control. Several NTD-inducing compounds have been identified, however the mechanism by which these environmental, pharmacological, and maternal toxicants disrupt proper neural tube closure is unknown. This project aimed to compare three developmental toxicants, known to cause NTDs, using the chick embryo. These compounds are: C2-ceramide (C2), fumonisin B1 (FB1), and valproic acid (VPA).

Ceramides and Fumonisin B1

Ceramide is a bioactive sphingolipid comprised of a sphingosine backbone linked to a fatty acid chain (Aburasayn et al., 2016). Ceramide synthesis occurs through several different pathways including de novo, salvage, and sphingomyelin hydrolysis (see Figure 2.1 B). Previous research in our lab identified C2 as a novel toxicant that increased risk of NTDs in exposed chicken embryos (Ross et al., 2019). Circulating plasma ceramides are elevated in overweight and obese individuals (Samad et al., 2006), creating a plausible link between the increased frequency of infants born with NTDs to overweight and obese women, who are 2-3x more likely
to have a baby with an NTD (McMahon et al., 2013; Huang et al., 2017). Interestingly, a potent ceramide synthase inhibitor and corn mold byproduct, fumonis B1 (FB1), has been associated with an increased risk for NTDs in mothers who consume high quantities of contaminated food (Gelineau-van Waes et al., 2009). Fumonis B1 is a structural sphingoid analogue, acting to competitively inhibit ceramide synthase function to reduce overall ceramide synthesis (He et al., 2006). Studies indicate that as much as 25% of world crops are contaminated with FB1-producing mold (Bullerman, 1996; Shephard et al., 1996). Contaminated corn has been associated with several diseases in animals, including equine leukoencephalomalacia, rabbit, lamb, and rat nephrotoxicity, and robust hepatotoxicity in most animal models evaluated (Humphreys et al., 2001).

Studies have indicated that this inhibitory effect of FB1 on ceramide production is the cause of NTD development (Wang et al., 1991; Constable et al., 2003) in both the chick (Zacharias et al., 1996) and the mouse embryo (Marasas et al., 2004). The buildup of metabolites (sphingosine and sphingonine) necessary for ceramide production has been reported in affected embryos treated with FB1 (Zacharias et al., 1996; Marasas et al., 2004). Free sphingoid bases are known to be cytotoxic and inhibit cell growth (Stevens et al., 1990). Fumonis B1 has also been shown to inhibit folate uptake by reducing concentrations of sphingolipid, including ceramides, that are necessary for the GPI-linked folate receptor functionality (Chatterjee et al., 2001).

*Valproic Acid*

While these toxicants have only been linked to NTDs in recent years, valproic acid (VPA), a commonly prescribed antiepileptic medication (AEM), has been known to increase the
risk for NTDs in offspring of mothers who take it during pregnancy for many years (Meadow, 1968). Epilepsy is a neurological disorder in which uncontrolled and unprovoked seizures occur regularly. While there is no cure, AEMs are commonly prescribed to prevent seizures. Each year in the United States, more than 25,000 babies are born to pregnant mothers who have diagnosed epilepsy (Meador et al., 2008). This pharmaceutical teratogen increases the risk for NTDs by 10-fold (Angus-Leppan and Liu, 2018; Wieck and Jones, 2018), while also increases risks for cardiac malformations, growth retardation, hypospadias, microcephaly, and facial/digit hypoplasia (Holmes et al., 2001; Bromley et al., 2010). Anticonvulsant medications are also prescribed to individuals suffering from migraines, depression, social disorders, fibromyalgia, and depression (Cascade et al., 2008), thus increasing the population of at-risk mothers.

Valproic acid is a wide-spectrum anticonvulsant medication that also carries mood-stabilizing properties (Wlodarczyk et al., 2012). Studies have explored possible mechanisms of VPA in various animal models, including zebrafish, xenopus, chick, rodents, dogs, and rhesus monkeys (summarized in (Wlodarczyk et al., 2012). Proposed mechanisms of VPA-induced NTDs primarily include 1) disruption of folate metabolism (Wegner and Nau, 1992) and 2) inhibition of HDAC, leading to increased H3 acetylation (Gotfryd et al., 2011) or reduced Wnt signaling (Eikel et al., 2006) in the developing embryo. However, sufficient evidence is lacking to support either as the primary mechanism of action.

**Comparing Toxicants**

While many genetic factors have been linked to NTDs in humans and animal models, they do not account for all cases. Environmental agents, including C2, FB1, VPA, and others (Robert and Guibaud, 1982; Sadler et al., 2002; Groenen et al., 2003; Missmer et al., 2006; Rasmussen et al., 2008; Chappell et al., 2009; Copp et al., 2013), disrupt proper development
resulting in defects. Even more compelling may be the compounded risk of certain genetic mutations and embryonic exposure to environmental toxicants. Therefore, a toxicological comparison was conducted to determine whether similar or distinct pathways were disrupted in embryos exposed to C2, FB1, or VPA, to identify cellular mechanisms responsible for NTDs. This study is crucial to understand how environmental toxicants impede proper neural tube closure, so future studies can identify genetic components that put babies at greater risk of NTDs and develop preventative measures that work for one or all toxicants.

Methods

Generally, all methodology utilized for these experiments was described in Chapter 2 (pages 19-23). Therefore, previously described methods are summarized here, and new methods are described in detail.

**P19 Cell Culture**

Undifferentiated P19 cells were cultured in alpha-modified MEM in an incubator at 37 °C. Neuronal differentiation was initiated with 1 µM retinoic acid and cultured to form aggregates from day 0-4, after which aggregates were collected and centrifuged. Pellets were dissociated in trypsin and cells were seeded at density of 40,000 cells/cm² on gelatin-coated tissue culture dishes and cultured until treatment on day 7.

**MDCK Cell Culture**

Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM media with 10% fetal bovine serum and 100 units/mL penicillin (Genesee), and 100 µg/mL streptomycin (Genesee), in a humidified incubator gassed at 5% CO₂, heated at 37 °C.
Embryo Culture

Eggs were obtained from local suppliers, incubated at 37 °C for 30-36 hours to obtain Stage HH6 embryos. Embryos were removed from the yolk using a filter paper disc and plated on 6-well culture plates treated with C2-ceramide (C2), fumonisin B1 (FB1), and valproic acid (VPA). Plates were placed in the incubator for up to 24 h.

Glutathione Redox Potential Analysis

Briefly, samples are collected in 5% perchloric acid containing 0.2 M boric acid (Sigma Aldrich) and 10 μM γ-Glu-Glu, as an internal standard. Samples were centrifuged to pellet protein and aliquots (300 μL) were derivatized. Aliquots (50 μL) were separated using a 3-aminopropyl column (5 μM; 4.6 mm × 25 cm) and detection was obtained by fluorescence monitoring with bandpass filters, 305–395 nm excitation and 510–650 nm emission. Quantitation was obtained by integration relative to the internal standard. To calculate the GSH redox potential (Eh), GSH and GSSG concentrations were used in the Nernst equation (Schafer and Buettner, 2001).

DCF Reactive Oxygen Species Assay

2’7’-Dichlorofluorescin diacetate (DCF-DA) is a reporter molecule used to visualize the generation of reactive oxygen species (ROS) in live cells or tissue. DCF-DA diffuses across lipid membranes where the acetyl group is cleaved by intracellular esterases, sequestering the 2’7’-dichlorofluorescin (DCF; Invitrogen) molecule within the cell. DCF is oxidized in the presence of ROS to generate a fluorescent compound.
**P19 Cells**

P19 cells were seeded at a density of $1 \times 10^5$ cells/well in a black with clear bottom 96-well plate and incubated with 100 $\mu$M DCF-DA in PBS for 45 min. Cells were carefully washed with PBS and then treated with different concentrations of C2 (6.25-800 $\mu$M). Results were obtained using a fluorescence spectrophotometer with an excitation wavelength of 475 nm and an emission wavelength of 525 nm.

**Whole Embryo Culture**

Embryos were incubated to HH8 and collected directly into a 5$\mu$M DCF-DA in 1x PBS solution and incubated at room temperature for 20 min. Embryos were then rinsed twice in 1x PBS and placed in a glass bottom cell culture dish with a control (0.5% DMSO in 1x PBS) or C2 (100 $\mu$M in 1x PBS) solution. To avoid bleaching of DCF fluorescence, embryos were imaged over a 16-minute period. An initial image was collected using Olympus IX-81 LED microscopy and CellSense; additional images were collected at the same settings at two-minute intervals for 16 min (9 images total). Relative percent change in measured DCF fluorescence was analyzed at four locations in the neural folds in each image of the series using ImageJ Software.

**Hanging Drop Assay**

The assay was performed as described by Kim et al., 2000, with minor modifications. Briefly, trypsinized MDCK cells were suspended at $2.5 \times 10^5$ cells/ml in DMEM (with 10% FBS, 1% Penstrep) and C2 (100 $\mu$M), FB1 (100 $\mu$M), or VPA (600 $\mu$M). Cell suspension drops (20 $\mu$L) were placed on the inner lid of a Petri dish and incubated for 0-4 h, and imaged at 0, 1, 2, 3, and 4 h. To image, drops were directly placed on a glass slide or triturated 10x through a 200 $\mu$L pipette and place don a glass slide. Three randomly selected areas of each drop were
imaged at 4x on an upright microscope for non-triturated (n=3) and triturated (n=3) conditions for each treatment. Cell aggregates were measured and binned according to size (Bin 1: clusters 1-10 cells, Bin 2: clusters 11-50 cells, or Bin 3: clusters>50 cells).

Mitochondrial Respiration Analysis

Briefly, stage 8 chick embryos were incubated for 12 h on 6-well agar plates, as previously described, with 1xPBS, 0.5% DMSO, 100 µM C2, 100 µM FB1, or 600 µM VPA. Embryos were collected and surrounding membranes removed in ice cold PBS. Respirometry was measured via O2 consumption using the Oroboros O2K Oxygraph (Innsbruck, Australia). Samples were collected in MiR05 respiration buffer and permeabilized at 37°C, followed by respiration measurement by a modified substrate-uncoupler-inhibitor-titration protocol described by Jheng et al., (2012). The chemical uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 2.5 µM) was added to determine full electron transport system (ETS) capacity. Following respirometry, embryo samples were collected from each chamber and analyzed for total protein concentration.

Statistics

Data was analyzed using Microsoft Excel 2016 and Prism 7 software. A confidence interval of 95% (α=0.05) was used as threshold for statistical significance. Data are presented as mean ± SEM, with sample size (n) specified in the corresponding figure. P19 Cells. Statistical significance was measured with Two-Way ANOVA and Dunnett’s multiple comparison tests (MTT) or Tukey’s multiple comparison tests (GSH and DCF). Chick Embryos. Statistical analysis was conducted with Two-Way ANOVA and Bonferroni (GSH, PH3, and LT) multiple
comparison tests, Unpaired T-test (embryo length, somite number) or Kruskal-Wallace and Welch’s t-tests (dose curve, NTD type).

Results

No Change in Cellular Viability and ROS Generation with VPA or FB1.

Undifferentiated P19 cells (n=3 for each treatment) were exposed to FB1 (0, 12.5, 25, 50, 100, 200, 400, or 800 µM) or VPA (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, or 2.4 mM) for 24 hours (n=4 at each dose tested; Figure 3.1 A-B). No significant decrease in viability was observed in cells under any concentration of FB1 or VPA (Figure 3.1 A-B). Viability is known to be reduced when concentrations exceed that of 100 µM C2 (page 24). Therefore, the working concentrations of 100 µM C2, 100 µM FB1, and 600 µM VPA are accepted as non-lethal.

Initial analysis of C2 exposure showed an increase in reactive oxygen species (ROS) generation in P19 cells with increasing concentrations (page 24). To determine whether FB1 and VPA also induce oxidative stress, undifferentiated P19 cells were exposed to various concentrations of FB1 (0, 25, 50, 75, 100, 150, or 200 µM) or VPA (0, 0.01, 0.75, 0.15, 0.3, 0.6, or 1.2 mM). Relative fluorescence was measured using 2’7’-dichlorofluorescin diacetate and compared to 1x PBS control. No difference in ROS generation with either treatment (Figure 3.1 C-D).
Figure 3.1: Cell Viability and ROS Generation Unaffected in Cell Culture.
Undifferentiated P19 neural precursor cell viability was assessed to determine the toxicity of fumonisin B1 (FB1) and valproic acid (VPA) on living cells. P19 cells were exposed to 0-800 µM FB1 or 0-2.4 mM VPA concentrations for 24 h (n=4). No significant change in viability was observed in FB1 or VPA treated cells (A, B). ROS generation was unaffected under any concentration of FB1 or VPA (C, D).
P19 Neuronal Differentiation Affected by C2, but Not FB1 or VPA

We aimed to study whether C2, FB1, or VPA altered the ability of P19 cells to differentiate. Neuronal differentiation is occurring at a rapid rate in the early neural tube and brain during embryonic development. Therefore, undifferentiated P19 cells were treated with retinoic acid on Day 0 (D0) to induce differentiation. Differentiating cells form aggregates between D0-D4 and begin to express the protein β3-Tubulin (β3-Tub), a marker of microtubule formation exclusive to neurons (Martellucci et al., 2019) and testis cells (Sullivan et al., 1986). Cultures were treated with 1xPBS, 0.5% DMSO, 100 µM C2, 100 µM FB1, or 600 µM on D0, 0.5, 1, 1.5, 2, 2.5, 3, or 3.5 (n=3 for all treatments). Cellular aggregates were then imaged and collected for β3-Tub analysis via Western Blot on D4 (Figure 3.2 A-C). Exposure to FB1 did not alter β3-Tub expression (Figure 3.2 A, D). Expression of β3-Tub in VPA-treated samples was decreased at D0 (p=0.0007), D0.5 (p=0.0019), D1 (p<0.0001), D1.5 (p<0.0001), D2 (p=0.00016), and D2.5 (p=0.0002), with normal expression at D3 and D3.5 (Figure 3.2 B, D). C2-ceramide exposure resulted in reduced β3-Tub in samples treated at D0 (p<0.0001), D0.5 (p<0.0001), D1 (p<0.0001), D1.5 (p<0.0001), D2 (p<0.0001), D2.5 (p<0.0001), and D3 (p<0.0001), with normal β3-Tub expression by in samples treated at D3.5 (Figure 3.2 C, D).

Epithelial Adhesion is Altered with Ceramide Treatment

Madin-Darby Canine Kidney (MDCK) cells are an established epithelial model for studying cellular adhesion. During neural tube closure, the neuroepithelium undergoes significant morphological changes to produce a neural tube from a flat sheet of cells. To assess whether adhesion is altered under treatment conditions, a hanging drop test was used. MDCK cells were suspended in medium and treated with C2 (100 µM), FB1 (100 µM), or VPA (600
Figure 3.2: P19 Neuronal Differentiation is Affected by VPA and C2. 
P19 cells underwent neuronal differentiation for 4 days (D0-D4). β3-tubulin (β3-Tub) expression was measured on D4 and normalized to control to determine effect on differentiation. FB1 had no effect on neuronal differentiation (A, D). VPA reduced overall β3-Tub expression in cells treated on D0 (p=0.0007), D0.5 (p=0.0019), D1.0 (p<0.0001), D1.5 (p=0.0005), D2 (p=0.0016), and D2.5 (p=0.0002), but had no effect on cells treated at D3 or D3.5 (B, D). C2 grossly reduced β3-Tub expression on D0 (p<0.0001), D0.5 (p<0.0001), D1.0 (p<0.0001), D1.5 (p<0.0001), D2 (p<0.0001), D2.5 (p<0.0001), and D3 (p<0.0001), with no effect on those cells treated on D3.5. Asterisks indicate significance between VPA and PBS control (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001). Carrots indicate significance between C2 and DMSO control (^= p≤0.05, ^^= p≤0.01, ^^^= p≤0.001, ^^^^= p≤0.00001).
Figure 3.3: MDCK Cell Adhesion Reduced with C2 Exposure.
The number of medium (10-50 cells) and large (>50 cells) aggregates was grossly reduced in MDCK cells cultured with 100 µM C2 over 4 h. Both trititated and untrititated samples were significantly affected ($p<0.0001$), while no difference was observed in cells treated with 600 µM VPA. A significant difference was observed in cell aggregate formation under 100 µM FB1 treatment conditions at 1 h ($p=0.0006$), with recovery by 4 h. Asterisks indicate significance (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.00001$).
µM). Twenty µL drops of cell suspension were incubated on the inner lid of a Petri dish and imaged at 0, 1, 2, 3, or 4 h post treatment, with or without trituration. Clusters were counted and binned based on the number of cells (Bin 1: 1-10 cells, Bin 2: 11-50 cells, or Bin 3: >50 cells). The data revealed that cell adhesion was significantly reduced in both triturated (p<0.0001) and un triturated (p<0.0001) MDCK cells exposed to 100 µM C2 after 4 h (Figure 3.3 A, B). FB1 reduced aggregate formation in non-triturated samples at 1 h (p=0.0006), with recovery by 4 h (Figure 3.3 C). No effect was observed under triturated conditions. Cells cultured with VPA showed no difference in adhesion when compared to PBS control in either treatment group (Figure 3.3 C, D).

Morphological Defects in Chick Embryos

Understanding that cell viability was predominantly unaffected in cell culture treated with various concentrations of C2, FB1, and VPA, we moved to the chick embryo model to assess effects of the toxicants during neural tube closure.

Using the EC culture method, as previously described in Chapter 2, we incubated HH8 chick embryos on 6 well agar plates with 1x PBS, and varying concentrations of fumonisin B1 (FB1; 50 µM, 100 µM, or 200 µM), or valproic acid (VPA; 300 µM, 600 µM, or 1.2 mM) for 24 h. Morphological and histological analysis was conducted to confirm neural tube defects (NTDs). Both cranial and caudal defects were observed across treatment groups and concentrations (Figure 3.4 A-D). The frequency of NTDs increased as the concentration of each toxicant increased (Figure 3.4 F,G), with penetrance rates of 42% with 50 µM FB1 (n=12), 50% with 100 µM FB1 (n=12; p=0.0422), 100% with 200 µM FB1 (n=12; p=0.0179), 40%
Figure 3.4: Observed Defects in Treated Embryos.
Affected embryos exposed to 100 µM fumonisin (FB1) or valproic acid (VPA) developed cranial and caudal defects like what is observed in human embryos with neural tube defects. Normal embryonic growth was observed in control embryos (A). Cranial defects included minor as well as more severe defects, including failed anterior neuropore closure (B) or neural fold fusion (C). Caudal defects like that shown in D and E were commonly observed in affected embryos (somite formation was unaffected at the level of the open folds). F. Fumonisin Dose Curve. Twenty-four hour incubation of chick embryos on control (1x PBS) or FB1-containing (50 µM, 100 µM, or 200 µM) agar plates resulted in increased NTD frequency at 100 and 200 µM doses. Control embryos experienced a NTD frequency of 6%, while exposure to 50 µM FB1 resulted in a 42% NTD frequency, 100 µM FB1 increased to 50%, and 100 µM FB1 resulted in 63% NTDs. 100 µM and 200 µM FB1-induced NTD rates were statistically significant when compared to control (p=0.0422, p=0.0179). G. Valproic Acid Dose Curve. Chick embryos were incubated as described in I, on control or VPA-containing (300 µM, 600 µM, 1.2 mM or 2 mM) agar plates. Five percent of control embryos had NTDs. The rate of NTDs increased to 40% at 300 µM (p=0.0256), 69% at 600 µM (p=0.0004), 76% at 1.2 mM (p<0.0001), and 100% at 2.0 mM (p<0.0001) of exposed embryos. All doses resulted in a significantly increased risk for NTDs. Asterisks indicate significance (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
penetrance with 300 µM VPA (n=15; p=0.0256), 69% penetrance with 600 µM with VPA (n=18; p=0.0004), 76% with 1.2 mM VPA (n=17; p<0.0001), and 100% with 2.4 mM (n=6; p <0.0001). A histological analysis to confirm presence and type of NTD type was conducted. Neural tube defects were categorized as cranial, caudal, and cranial + caudal defect, depending on location within the embryo. Cranial defects occurred anywhere between the anterior neuropore and the 10th somite, caudal defects between the 11th somite and posterior neuropore, and cranial + caudal defects were embryos with a defect in both region. Analysis of NTD type revealed no significant difference observed across treatments (data not shown).

Embryo development is associated with both structural development and overall growth. We assessed the rate of growth of control and treated embryos, as measured by cranial to caudal
Figure 3.5: Somite Development and Embryo Growth.
The number of somite pairs each affected (NTD) or normal (no NTD) embryo developed during 24 h incubation with C2 (100 µM), FB1 (100 µM), or VPA (600 µM) was compared to control (0.5% DMSO or 1x PBS). Normal and affected embryos treated with FB1 developed significantly fewer somites (p<0.0001, 0.0017 respectively) when compared to control (C, D). No difference in somite pair developed was observed in normal or affected C2 or VPA treated embryos (A-D). Embryo length (cranial-caudal) differed in C2 treated affected embryos (p=0.0013; F). FB1-treated embryos were significantly shorter with (p=0.0449; G) or without NTDs (p=0.0115; H). Embryos exposed to VPA were significantly longer than control (0.0057; G). Asterisks indicate significance (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
length and somite pair development after 24 h incubation (Figure 3.5 E-H). Control PBS-treated embryos grew an average of 6.08 mm (n=35). Embryos exposed to 100 µM FB1 that were normal (no NTD) grew an average of 5.28 mm (n=14; p=0.0013), and those with NTDs grew an average of 5.314 mm (n=7; p=0.0115). Normal embryos exposed to 600 µM VPA grew 7.14 mm (n=4; p=0.0057). NTD-affected embryos exposed to VPA grew 5.71 mm (n=5; p=0.2793).

DMSO-treated embryos without NTDs grew an average of 5.57 mm (n=18), while those with NTDs grew an average of 5.44 mm (n=10). Those embryos exposed to 100 µM C2 that developed normally grew an average of 5.66 mm (n=20; p=0.9263). C2-treated embryos with NTDs grew only 4.93 mm (n=18; p=0.0479).

**Cellular Proliferation Unaffected, While Cell Death is Increased in Chick Embryos**

To determine whether FB1 or VPA altered cell proliferation or death in treated embryos, we analyzed phospho-histone 3 (PH3) and activated lysosomal (LysoTracker Red; LT), respectively.

To assess proliferation, stage 8 chick embryos were incubated on FB1 or VPA agar plates for 24 h and stained with PH3 antibody. Embryo (n=2-4) sections were grouped into five regions of interest: Head, Otic, Upper Spinal Cord (USC), and Lower Spinal Cord (LSC), and caudal. The count, total and within the neural tube, of PH3+ cells were measured for each section and recorded. As visualized in Figure 3.6 and Table 3.1, most regions showed a statistically significant reduction in PH3+ cell after FB1 and VPA exposure, with variability across sections and embryos. Total PH3+ cell count (Figure 3.6 B) was significantly less in both treatment groups when compared to control in all groups: Head (p<0.0001, p=0.002), Otic (p<0.0001, 0.0037), USR (p<0.0001, both), LSR (p=0.0001; p=0.0002), and Caudal (p=0.0002;
Figure 3.6: Embryonic Cell Proliferation is Downregulated with FB1 and VPA Exposure. Expression of PH3 was evaluated in embryo sections to determine proliferation, both within the neural tube (A) and the entire section (B). No significant difference was observed in the number of PH3+ cells within the neural tube across treatments (A). However, the number of PH3+ positive cells in the whole section of each region of the embryo was significantly reduced in VPA- and FB1- treated embryos. Asterisks indicate significance between VPA and PBS control (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.00001). Carrots indicate significance between FB1 and PBS control (^ = p ≤ 0.05, ^^ = p ≤ 0.01, ^^^ = p ≤ 0.001, ^^^^^ = p ≤ 0.00001).
Table 3.1: Comparison of Embryonic Cellular Proliferation with PBS, FB1, or VPA Treatment. Tissue sections were analyzed for PBS-, FB1-, and VPA-treated embryos to obtain PH3+ (marker for M phase) cell counts. Sections were grouped by region based on location along the A-P axis in five groups: Head, Otic, Upper Spinal Cord (USC), Lower Spinal Cord (LSC), and Caudal. Due to high variability, an average PH3+ cell count is reported for all sections and an average within the neural tube (NT). Significance was observed in total count for the section across treatments and regions, however no significance was observed in count for neural tube. Significant P values bolded.

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Figure 3.7: Valproic Acid Increases Cellular Apoptosis in Treated Embryos. Analysis of acidic lysosomal staining in PBS, 100 µM FB1 or 600 µM VPA treated embryos (n=2-3 per treatment) was performed to determine whether cell death was increased globally or within the neural tube. Increased total punctae staining (p<0.0001), as well as punctate within the neural tube (p=0.0002) was observed when VPA was compared to both PBS and FB1. No significant difference was observed in FB1 treated embryos when compared to control. Asterisks indicate significance (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
The number of proliferating cells within the NT was decreased in VPA treated embryos when compared to control in the Head (p=0.0185), but not in any other group (Figure 3.6 A). Proliferation was decreased in the NT of embryos treated with FB1 in the Head (p<0.0001), Otic (p=0.0203), and USR (p=0.034) when compared to control. Ceramide treatment showed a global reduction in cell proliferation (page 28). LysoTracker Red (LT), was used to visualize activated lysosomes, a marker for cell death, in embryos cultured in PBS control (n=2) and FB1 (n=3) or VPA (n=3) for 24 h. The number of LT punctae were quantified in histological sections (Figure 3.7). When comparing treatments to control, no difference was observed in the overall number of LT-positive acidic lysosomes in FB1-treated embryos. VPA induced significantly more LT-positive staining, both within the NT (p=0.0002) and globally in the embryo (p<0.0001) when compared to control and FB1. Cranial vs. caudal sections and open vs. closed neural tubes also showed no difference (data not shown).

**Glutathione Redox Potential in Chick Embryos is Altered Differentially in Response to C2, FB1, and VPA Treatment**

Chick embryos (HH8) were cultured with 100 µM FB1 or 600 µM VPA for 24 hours. Samples were collected at 0, 1, 2, 4, 6, 8, 12, or 24 h and assessed for glutathione (GSH) redox potential (Eh) utilizing HPLC analysis.

Glutathione redox potential significantly and differentially affected in the chick embryo under FB1 and VPA treatment. Fumonisin B1 exposure resulted in a significant shift to a more reduced tissue redox state in samples collected at 1 h (p=0.0452), 4 h (p=0.0011), 8 h (p<0.0001), and 12 h (p<0.0001), with recovery to a normal state by 24 h (Figure 3.8A). Samples at 2 h were also more oxidized, but not significant (p=0.1148). This shift appears to be
Figure 3.8: Embryonic Glutathione Redox Potential Analysis 24 hours.
A. The glutathione (GSH) redox potential ($E_h$) of chick embryos exposed to FB1 (100 µM) was significantly reducing when compared to that of control at 1 h ($p=0.0452$), 4 h ($p=0.0011$), 8 h ($p<0.0001$), and 12 h ($p<0.0001$). B. The tissue of VPA (600 µM) exposed embryos was significantly more reduced at 1 h ($p=0.0004$) and 2 h ($p=0.0182$), then the tissue becomes more oxidized at 4 h ($p=0.0051$) and 8 h ($p=0.0233$). Carrots indicate significance between FB1 and PBS control ($^\wedge= p\leq 0.05$, $^{\wedge\wedge}= p\leq 0.01$, $^{\wedge\wedge\wedge}= p\leq 0.001$, $^{\wedge\wedge\wedge\wedge}= p\leq 0.00001$). Asterisks indicate significance between VPA and PBS control ($*= p\leq 0.05$, $**= p\leq 0.01$, $***= p\leq 0.001$, $****= p\leq 0.00001$).
an opposite response when compared to embryonic tissue exposed to C2, where an oxidizing shift was observed between hours 1-8 post treatment (Ross et al., 2019). Valproic acid-exposed embryos resulted in an initial shift to a more reduced state at 1 h (p=0.0039), with a shift to a more oxidized state at 4 h (p=0.0402), when compared to control (Figure 3.8 A).

*Cellular Redox State is Grossly Affected by C2 Exposure, but Not FB1 or VPA*

We previously reported that while the cellular redox state of undifferentiated P19 cells was unaffected, that of neuronally differentiated cells was significantly oxidized in response to C2 (100 µM) treatment (Ross et al., 2019). Therefore, we similarly treated P19 cells with FB1 (100 µM) or VPA (600 µM) to determine the individual effects of these drugs on the cellular redox state of P19 cells under each condition (undifferentiated or differentiated; n=2-3 for all treatments). The cellular redox state of undifferentiated cells was unaffected when exposed to FB1 (Figure 3.9 A). However, VPA induced an oxidizing shift in the GSH Eh at 1 h (p<0.0001), 2 h (p<0.0001), 4 h (p<0.0001), 6 h (p=0.0001), 8 h (p=0.0002), 12 h (p<0.0001), and 24 h (p<0.0001; Figure 3.9 B).

Differentiated P19 cell exposure to VPA resulted in a more oxidized GSH Eh at 1 h (p=0.0379) and 24 h (p=0.0466), while exposure to FB1 resulted in an oxidizing shift in the GSH Eh at 24 h (p=0.0301). The GSH Eh was not significantly different at any other time point under either treatment condition (see Figure 3.9 C, D). These redox potential shifts under VPA and FB1 treatments indicate a differential response in the tissue when compared to all treatments.

*Mitochondrial Functionality was Altered in Embryos Exposed to C2, but Not FB1 or VPA*

To determine whether these shifts in tissue redox state were a result of toxicant-induced mitochondrial dysfunction, embryos were cultured on drug agar plates treated with 1x PBS,
DMSO (0.5%), C2 (100 µM), FB1 (100 µM), or VPA (600 µM) for 12 h before collected and analyzed for mitochondrial respiration using the Oroboros O2K oxygraph (n=3, with 4-5 embryos pooled per sample). Respiration was measured as oxygen (O₂) flux at full uncoupling with FCCP and compared to control. Mitochondrial respiration was unaffected by VPA treatment when compared to PBS control, while FB1 caused a slight reduction in overall O₂ flux (Figure 3.10). C2 exposure increased O₂ consumption when compared to DMSO control (p=0.0207).
Figure 3.9: Cellular Glutathione Redox Potential Analysis 24 hours.
The glutathione (GSH) redox potential (E_h) of undifferentiated P19 cells was unaffected by FB1 exposure over 24 h (A). Treatment with VPA resulted in a significantly oxidizing shift in the redox potential of undifferentiated P19 cells from 1-24 h (B). Neuronally differentiated P19 cells were unaffected by FB1 until 24 h, where the GSH E_h became more oxidized (C). Similarly, an oxidizing shift in the GSH E_h was observed at 2 h and 24 h in differentiated P19 cells treated with VPA (D). Asterisks indicate significance between VPA and PBS control (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001). Carrots indicate significance between C2 and DMSO control ( ^= p≤0.05, ^^= p≤0.01, ^^^= p≤0.001, ^^^^^= p≤0.00001).
Figure 3.10: Embryonic Mitochondrial Respiration is Increased by C2-ceramide. Chick embryos were exposed to 100 µM C2, 100 µM FB1, or 600 µM VPA for 12 hours and collected for mitochondrial respirometry (n=3, with 4-5 embryos pooled per sample). Oxygen (O2) flux was measured after full uncoupling following the addition of FCCP and normalized to control. Respiration was unaffected by VPA and FB1 when compared to PBS control, but increased in C2-treated embryos when compared to DMSO control (p=0.0207). Graphs represent comparison to normal flux of 100%. Asterisks indicate significance (*= p≤0.05, **= p≤0.01, ***= p≤0.001, ****= p≤0.00001).
Discussion

This study was designed to compare three neural tube defect (NTD)-inducing toxicants using the chick embryo to determine whether similar or differential mechanisms were employed to result in the same defect. Presently, the only effective pre-treatment known to reduce the risk for NTDs is pre- and perinatal consumption of folic acid (FA). While effective in reducing NTD frequencies by up to 70%, there are still thousands of cases each year in which FA is ineffective and babies are born with these life-threatening defects.

A comparative analysis such as this one employs an inexpensive model to evaluate the effects of environmental and pharmaceutical toxicants during development. We confirmed that while working concentrations were not cytotoxic and did not induce reactive oxygen species (ROS) (Figure 3.1), an increased number of NTDs occurred in embryos exposed to C2-ceramide (C2), fumonisin B1 (FB1), and valproic acid (VPA) (Figure 3.4). Morphological analysis revealed that FB1 exposure retarded overall growth (as measured by embryo length) and somite pair development in both affected and normal embryos (Figure 3.5 C, D, F, G). C2 also reduced the number of somite pairs that formed in embryos with NTDs (Figure 3.5 F). Interestingly, normal embryos exposed to VPA grew significantly longer than control embryos (Figure 3.5 G). We explored various cellular responses to determine whether these differential responses were also observed in other readouts, including cellular differentiation, adhesion, death, proliferation, redox state changes, and mitochondrial respiration.

As the neural tube closes, epithelial modifications and adhesions are required to unite and fuse the neural tube and non-neural ectoderm, resulting in an enclosed structure. We examined the effects of C2, FB1, and VPA on MDCK cell ability to form and maintain tight adhesions. While FB1 and VPA had no effect, C2 significantly reduced the number of aggregates that
formed (both with and without trituration), suggesting this lipid molecule may reduce cellular adhesion within the neural tube. In recent years, experimental misexpression of tight junction proteins, including claudins (Baumholtz et al., 2017), has been linked to increased frequency of NTDs, indicating the formation and maintenance of these tight bonds is critical for proper neural tube closure.

As the neural tube is closing, the embryo undergoes rapid growth, during which cellular proliferation, differentiation, apoptosis, and metabolic activities are orchestrated within strict boundaries. To model neuronal differentiation, like that occurring in the neural tube, a P19 embryonic carcinoma cell line used. Data revealed significant reduction in differentiation, as measured by β3-tubulin expression, by C2 and VPA exposure in the early stages of differentiation (D0-D2.5; Figure 3.2). Evaluation of metabolic function under toxicant exposure revealed a reduction in overall mitochondrial respiration in response to FB1 and C2 (Figure 3.9).

Oxidative stress and cell death are often a result of poor mitochondrial respiration. Therefore, we examined glutathione (GSH) redox potential ($E_h$) as a readout for tissue redox state. Undifferentiated and differentiated P19 cells were exposed to C2, FB1, or VPA for up to 24 h and collected for GSH $E_h$ analysis. The GSH $E_h$ of undifferentiated cells underwent an oxidizing shift at each time point from 1-24 h in response to VPA exposure, but not FB1 (Figure 3.8 B) or C2 (Figure 2.3 A). Once differentiated, P19 cells exposed to C2 undergo an oxidizing shift in the redox state (Figure 2.3 B), however FB1 had no effect until 24 h (Figure 3.8 C). VPA caused an oxidizing shift at 2 h and 24 h, but it was not sustained at intermediate timepoints within the treatment window.

In the embryo, a different response was observed in the GSH $E_h$. Embryos treated with FB1 experienced reducing stress from 1-12 h, followed by recovery to a normal state. VPA
induced an initial oxidizing shift at 2 h, followed by a reducing shift in the redox state at 4 h and recover to normal by 8 h (Figure 3.8 A). This differential response in a controlled P19 cell line and a complex embryo is not a novel finding, as C2 displayed an oxidizing shift in the embryo from 1-8 h, followed by a recovery to normal. However, P19 GSH $E_h$ in differentiated cells became oxidized later (24 h). An examination of cellular apoptosis, measured by activated lysosomes, revealed an increased in overall cell death in VPA-treated embryos (Figure 3.7). C2 and FB1 exposure did not result in greater cell death. However, overall cellular proliferation was reduced across treatments (Figure 3.6).

This unique response in the redox state of embryos treated with C2, FB1, or VPA may be the result differential protein modification, resulting in downstream effects (genetic, functional, or biochemical) that can lead to disrupted neural tube closure. Every protein has a redox midpoint (Timme-Laragy et al., 2013). If a reducing or oxidizing shift, like those observed in FB1- or C2-treated embryos, occurs during critical windows of development, proteins may be modified as a result. These modifications be they genetic, functional, or biochemical, lead to disrupted neural tube closure.

In short, at least one differential response was observed in each analysis, suggesting that when considering developmental toxicology and the resulting defects, a “one size fits all” mechanism in NTD formation is unlikely. Environmental and maternal toxicant or pharmaceutical exposures may vary globally, putting certain groups at greater risk for having children with these devastating and expensive defects. Therefore, it is imperative that descriptive analyses, such as this, be performed to better understand toxicological effects on cells and tissues throughout development to develop better preventative measures.
Future studies should aim to include additional toxicants known to induce NTDs, including heat, retinoic acid, and hyperglycemia, to expand the current understanding for each of these teratogens. RNA-sequencing may reveal changes in gene regulation in response to specific mechanisms explored here, or lead to the identification of additional assays to broaden our knowledge about these toxicants. These studies will inform scientific and medical professionals on the best approaches to reduce the risk for NTDs in prospective mothers.
CHAPTER 4: Gene Expression Changes in Response to Developmental Toxicants Known to Induce Neural Tube Defects: A Comparison of RNA-Sequencing Profiles of Chicken Embryos Treated with C2-Ceramide, Fumonisin B1, and Valproic Acid

Introduction

The early embryo begins as a flat sheet of cells that thickens and raises bilaterally along the AP axis. These ridges undergo mechanical bending to unite at the hindbrain and adhere dorsally from head to tail to generate the enclosed neural tube (NT). In the chick embryo, these structural changes occur within 50 hours of fertilization. The orchestration of these cellular changes during development relies on time- and cell-specific gene expression. RNA sequencing technologies have advanced significantly over the last decade, providing an invaluable tool to identify gene expression profiles throughout development. This has allowed scientists to better understand how genetic mutations negatively impact embryo growth and morphology.

More than 300 genes have been linked to failed NT closure during early development, resulting in neural tube defects (NTDs) in the mouse model (Harris and Juriloff, 2007; Harris and Juriloff, 2010; Juriloff and Harris, 2018). RNA sequencing projects have evaluated specific gene families, localized gene groups, and transcriptomic changes in affected human and mouse models (Gustavsson et al., 2008; Krupp et al., 2012; Dong et al., 2016; Yu et al., 2017; Kindt et al., 2018) Many of these genes are involved in key developmental processes such as cell polarity, division, migration, proliferation, patterning, metabolism, and nutrient transport. In human pregnancies, if one child is affected with a NTD, the rate of risk for siblings is 2-5% (Rampersaud et al., 2006). However, the evidence pointing to genetic mutations as the primary source of these congenital defects is minimal, suggesting that multifactorial conditions lead to failed NT closure.
Gene regulation can be altered by more than mutations. Environmental toxicant exposure during development can up- or down-regulate the expression of genetic information. Previous studies in our lab have compared three different teratogens, or developmental toxicants, known to induce NTDS: C2-ceramide (C2), fumonisin B1 (FB1), and valproic acid (VPA). We characterized several differential responses in embryos exposed to each toxicant (summarized in Table 4.1). Therefore, to determine whether C2, FB1, and VPA differentially alter gene regulation, RNA-sequencing is necessary.

Methods

Embryo Culture

As described in Chapter 2, fertilized chicken eggs were obtained from local vendors and incubated at 38.5°C for 28-32 h to Hamburger & Hamilton (HH) stage 8. Embryos were collected using the early chick (EC) culture method described Chapman et al. (2001). Briefly, an egg was cracked into a petri dish, thick albumin was removed using a Kimwipe so a filter paper disc could be placed around the embryo. The embryo was then cut from the vitelline membrane and suspended embryo from the yolk. A second filter paper disc was placed on the ventral side to create a sandwich. Embryos were subsequently plated on control or drug treated six-well agar plates (as described below). Embryos were incubated on the plates for a specified time before being removed into fresh 1x PBS for imaging.

Embryo Plate Preparation

Embryos were collected as described in Chapter 2 (page 20). Briefly, stage 8 chick embryos were collected from the yolk using a filter paper disc, then placed on drug or control
plates and incubated at 37 °C for 6 h. 6-well plate preparation for chicken embryo culture was as follows:

**Drug Plate**

A stock solution was made for each drug: C2 (Cayman Chemical) stock solution of 20.91 mM (1 mg/140 μL DMSO), FB1 (Cayman Chemical) stock solution of 20 mM (1 mg/67 μL 1xPBS), or VPA (Sigma-Aldrich) stock solution of 40 mM (1mg/150 μL 1xPBS). Each stock solution was diluted in 2 mL 1%BSA/PBS with PenStrep (1:1000) to working solution concentrations: 100 μM C2, 100 μL FB1, and 600 μL VPA. 6 mL thin albumin was added, followed by 6 mL agar (0.18 g BactoAgar in 6 mL saline) to produce 14 mL homogenized drug solution. 2 mL of the solution was added to each well of a 6 well plate and refrigerated to solidify.

**Control Plate**

70 μL Dimethyl Sulfoxide (DMSO; Millipore) or 70 μL 1xPBS was added to 2 mL 1%BSA/PBS with PenStrep (1:1000). 6 mL thin albumin was added, followed by 6 mL Agar (0.18 g BactoAgar in saline) to produce 14 mL homogenized drug solution. 2 mL of the solution was added to each well of a 6 well plate and refrigerated to solidify.

**RNA Extraction**

Embryos (n=3 per treatment) were cultured on 6 well plates treated with C2, FB1, VPA, DMSO, or PBS for 6 h prior to being removed from surrounding membranes in ice cold 1xPBS before being collected into 200 μL Trizol (Zymo Research). Samples were placed in the freezer overnight before RNA extraction was performed using Direct-zol RNA Miniprep kit (Zymo Research). RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies). RNA
(1.5 mg) from each sample was converted to mRNAseq library at the CvDC RNA Expression Core using Illumina Tru-seq kit (Illumina) and sequenced via Rapid Mode on a HiSeq 2500 (Illumina). Using Noalign (Novacraft Technologies) sequences were aligned to gallus gallus genome GRCg6a. Differential expression between drug and control groups (C2 v DMSO; FB1 and VPA v PBS) at 6 h post-treatment using the DSeq package in R Studio.

Results

*Differentially Expressed Genes in Response to C2, FB1, or VPA Exposure in the Chick Embryo*  
Embryos were incubated on 6 well agar plates with 1x PBS, DMSO (0.5%), C2 (100 µM), FB1 (100 µM), or VPA (600 µM) for 6 h, a timepoint specifically chosen based to capture the earliest changes in gene expression, rather than downstream gene-regulatory events. Individual embryos (n=3 per treatment) were then collected for RNA extraction and analysis. Sample libraries were combined by treatment and compared to control for expression analysis. There were 9,417, 8,939, and 10,873 genes measured in library C2 vs DMSO, FB1 vs PBS, and VPA vs PBS, respectively. A GO analysis was performed to classify the genes. No significant differences were observed across treatment groups.

*Genes of Interest from C2 vs DMSO Library*  
The expression of 16 differentially expressed genes (DEGs) was reported as significantly altered in the C2 vs DMSO library. A significant reduction in the expression of 11 of these DEGs and significant overexpression of 5 DEGS is summarized in Table 4.1. The 11 downregulated DEGs include TXNIP, GNAO1, DDIT4, PRKAR1β, DNAH10, TERB1, LRRC8B, CKB, ADNP2, and 2 novel genes. Those DEGs upregulated under C2 conditions include
SLC38A2, DNAJC12, SLC6A6, CAB39L, and one novel gene. Those genes of initial interest include TXNIP, DDIT4, ADNP, CKB, and SLC38A2.

Thioredoxin interacting protein (TXNIP) expression is significantly downregulated in embryos exposed to C2. TXNIP is a stress-induced regulator of cellular metabolism and thioredoxin antioxidant activity, including thioredoxin (Trx) 1 and 2 (Shalev, 2014). Under stressful oxidative conditions, TXNIP moves from the nucleus to the mitochondria where it can oxidize Trx 2, leading to cellular apoptosis (Saxena et al., 2010). One study reported decreased expression or KO of TXNIP led to reduced cellular respiration, shifting mitochondrial function from oxidative phosphorylation towards anaerobic glycolysis (Yoshioka et al., 2012). We observed that embryonic redox state was significantly oxidized (page 31) and mitochondrial respiration was negatively impacted by C2 treatment (page 67). Further study is necessary to determine whether TXNIP is responsible for these activities, and whether it is directly or indirectly regulated by ceramide exposure.
Table 4.1: Gene Table of Significant DEGs from Ceramide vs DMSO RNA-seq Library

Expression of 16 genes was significantly altered in embryos exposed to C2-ceramide when compared to DMSO control. Expression of 11 DEGs was reduced (above bolded line), while 5 DEGs were overexpressed.

<table>
<thead>
<tr>
<th>Ensembl Gene ID</th>
<th>Gene Name</th>
<th>Gene Product</th>
<th>2-Fold Change</th>
<th>P-value</th>
<th>Padj</th>
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<td>ENSGALG00000005957</td>
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<td>0.000274</td>
<td>0.044299</td>
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<td>ENSGALG000000048409</td>
<td>TXNIP</td>
<td>Thioredoxin interacting protein</td>
<td>-1.61687</td>
<td>2.66E-10</td>
<td>7.43E-07</td>
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<td>ENSGALG00000003163</td>
<td>GNAO1</td>
<td>G protein subunit alpha 01</td>
<td>-1.40904</td>
<td>0.000125</td>
<td>0.029876</td>
</tr>
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<td>ENSGALG000000041459</td>
<td>DDIT4</td>
<td>DNA damage inducible transcript 4</td>
<td>-1.28995</td>
<td>0.00016</td>
<td>0.033579</td>
</tr>
<tr>
<td>ENSGALG00000003675</td>
<td>PRKAR1B</td>
<td>Protein kinase cAMP-dependent type I regulatory subunit beta</td>
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<td>1.26E-06</td>
<td>0.001174</td>
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<tr>
<td>ENSGALG00000006644</td>
<td>Novel</td>
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<td>0.000144</td>
<td>0.032663</td>
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<tr>
<td>ENSGALG000000043806</td>
<td>DNAH10</td>
<td>Dynein axonemal heavy chain family 10</td>
<td>-1.14354</td>
<td>1.65E-05</td>
<td>0.006943</td>
</tr>
<tr>
<td>ENSGALG00000005214</td>
<td>TERB1</td>
<td>Telomere repeat binding bouquet formation protein 1</td>
<td>-1.08862</td>
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<td>0.029876</td>
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<tr>
<td>ENSGALG00000006167</td>
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<td>0.001763</td>
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<td>ENSGALG00000011511</td>
<td>CKB</td>
<td>Creatine kinase B</td>
<td>-1.028</td>
<td>3.19E-07</td>
<td>0.000383</td>
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<tr>
<td>ENSGALG00000035669</td>
<td>ADNP2</td>
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<td>8.35E-05</td>
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<td>SLC38A2</td>
<td>Solute carrier family 38 member 2</td>
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<tr>
<td>ENSGALG00000002769</td>
<td>DNAJC12</td>
<td>DnaJ heat shock protein family (Hsp40) member C12</td>
<td>1.084619</td>
<td>9.75E-05</td>
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<td>ENSGALG00000006425</td>
<td>SLC6A6</td>
<td>Solute carrier family 6 member 6</td>
<td>1.231983</td>
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<td>2.54E-08</td>
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<td>ENSGALG00000017005</td>
<td>CAB39L</td>
<td>Calcium binding protein 39 like</td>
<td>1.349427</td>
<td>6.99E-06</td>
<td>0.004188</td>
</tr>
</tbody>
</table>
DNA damage inducible transcript 4 (DDIT4) is a known mTOR repressor. DDIT4 has been implicated in neuronal differentiation and is generally upregulated in response to cellular responses, including heat shock (Wang et al., 2003), radiation (Ellisen et al., 2002), hypoxia (Shoshani et al, 2002), and energy stress (Sofer et al., 2005). Endoplasmic reticulum stress (Wang et al., 2003) and glucocorticoids (Molitoris et al., 2011) are also known to upregulate DDIT4. Conversely, Gordon et al., (2016) reported DDIT4 is downregulated in response to resistance exercise, nutrient consumption, mTOR suppression, and testosterone. Decreased DDIT4 expression is also associated with reduced expression of pluripotent genes, including Oct4, Klf4, and Nanog (Gharibi et al., 2016).

Activity-dependent neuroprotective protein (ADNP) is a transcription factor associated with neuronal differentiation, cell growth regulation, and has been characterized in the neural tube in the mouse embryo, and when knocked out results in cranial NTDs (Pinhasov et al., 2003). ADNP is necessary for proper neural tube closure, regulating genes such as Wnts (Sun et al., 2020), Oct4 and Pax6, and is associated with neurogenesis, organogenesis, and lipid transport (Mandel et al., 2007).

Less is known about CKB and SLC38A2. However CKB has been characterized in highly oxidative tissues as a catalyst for transferring phosphates from ATP to creatine, and is known to be expressed in the brain of embryos (Roy et al., 2013). This suggests a possible link to reduced respiration in C2-treated embryos. SLC38A2 or SNAT2 is a solute transporter found in embryonic stem cells associated with the uptake of nutrients necessary for differentiation (Tan et al., 2016).
Genes of Interest from FB1 vs PBS Library

In the FB1 vs PBS library, 20 DEGs were significantly over (15 genes) or under (5 gene) expressed (see Table 4.2). Under FB1 treatment, the expression those significantly upregulated DEGs include PTBP2, PAPD7, UTP15, IRX3, PLAA, IPO11, ZFAND5, DMXL1, DIMT1, CERT1, TRIM23, RPPL25L, FST, SLC6A6, and 1 novel gene. HOXB3, SEPT5, ALDH1L2, HERPUD1, and CDX2 were all downregulated in response to FB1. Of these, CDX2, HOXB3, IRX3, and FST have been linked to abnormal embryo and brain development, while HERPUD1 and CERT1 have been linked to oxidative stress. These genes are of interest and should be examined further.

CDX2, HOXB3, and IRX3 are transcription factors that have been characterized in the developing embryo and involved in neural tube patterning and closure. CDX2 is necessary for axial elongation (Chawengsaksophak et al., 2004), cell polarity (Savory et al., 2011), and neural tube closure (Zhao et al., 2014). Interestingly, CDX genes are upstream to HOX genes, including HOXB3 (Bel-Vialar et al., 2002). HOXB3 is known to regulate hindbrain patterning in the early embryos (Trainor and Krumlauf, 2000). IRX3 is expressed in neural progenitor cells (Bellefroid et al., 1998), is associated with neuronal differentiation (Briscoe et al., 2000), and responsible for forebrain-midbrain boundary formation and patterning (Anselme et al., 2007). Follistatin (FST) is a BMP-inhibitor expressed throughout early embryo development and patterning. One study found that when neural cells were exposed to FST, cell fate switched from a dorsal to a ventral neuronal fate (Liem et al., 2000). FST is also expressed during somite differentiation in the chick (Amthor et al., 1996).

HERPUD1 is localized to the endoplasmic reticulum (ER) and is upregulated in response to ER stress (Ho and Chan, 2015). Most research surrounding HERPUD1 has focused on cancer.
Table 4.2: Gene Table for Significant DEGs from Fumonisin vs PBS RNA-seq Library. Expression of 20 genes was significantly altered in embryos exposed to Fumonisin B1 when compared to PBS control. Expression of 5 DEG was reduced (above bolded line), while 15 DEGs were overexpressed.

<table>
<thead>
<tr>
<th>Ensembl Gene ID</th>
<th>Gene Name</th>
<th>Gene Product</th>
<th>2-Fold Change</th>
<th>P-value</th>
<th>Padj</th>
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<tr>
<td>ENSGALG00000034983</td>
<td>CDX2</td>
<td>Caudal type homeobox 2</td>
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<td>ENSGALG00000001220</td>
<td>HERPUD1</td>
<td>Homocysteine inducible ER protein with ubiquitin like domain 1</td>
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<td>5.43E-05</td>
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</tr>
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<td>ENSGALG00000012691</td>
<td>ALDH1L2</td>
<td>Aldehyde dehydrogenase 1 family member L2</td>
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<td>ENSGALG0000001688</td>
<td>SEPT5</td>
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<tr>
<td>ENSGALG00000043219</td>
<td>HOXB3</td>
<td>Homeobox B3</td>
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<tr>
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<td>PTBP2</td>
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<td>0.000112</td>
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</tr>
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<td>ENSGALG00000013061</td>
<td>PAPD7</td>
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<td>ENSGALG00000013510</td>
<td>UTP15</td>
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<tr>
<td>ENSGALG00000042045</td>
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<td>ENSGALG0000001782</td>
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<td>ENSGALG00000014908</td>
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<td>7.36E-10</td>
</tr>
</tbody>
</table>
biology and ER stress, with little to no information regarding the role it may play in embryonic development. *CERT1* is a ceramide transporter responsible for trafficking ceramides from the ER to the Golgi apparatus for glucosylceramide generation (Hanada et al., 2009). Because FB1 is known to reduce ceramide generation, it may be that *CERT1* expression is upregulated in embryos exposed to FB1 as an attempt by the embryo to try and traffic what little ceramide exists in the ER to the Golgi for necessary modifications and use.

**Discussion**

RNA-sequencing has greatly impacted our understanding of both normal and abnormal embryonic growth and development. This study presents a list of novel genes that are differentially expressed under conditions of toxicants known to induce neural tube defects (NTDs), the second most common defect in the world.

In this study, five libraries were generated: two control (1xPBS and 0.5% DMSO) and three treated (100 µM ceramide, 100 µM fumonisin B1, and 600 µM valproic acid). A similar number of total genes was measured in each treatment library. A Gene Ontology analysis was performed on each treatment library to categorize genes based on their function within the cell, showing no significant difference across libraries.

Treatment libraries were compared to their respective controls and differentially expressed genes (DEGs) were measured, with no significantly affected pathways were identified for any library. Expression profiles of DEGs identified in the Ceramide vs DMSO library, revealed 11 genes of interest (GOIs) that were significantly downregulated and 5 GOIs were significantly upregulated (Table 4.1). Our lab is interested in characterizing and evaluating five of these genes in the chick embryo to determine whether they play a role in neural tube closure. These genes include *TXNIP, DDIT4, ADNP, CKB, and SLC38A2*. The expression of 5 GOIs
from the Fumonisin vs PBS library were significantly downregulated, with 15 GOIs showing significant upregulation (Table 4.2). We are interested in elucidating the roles of FB1 regulation of \textit{CDX2, HOXB3, IRX3, UTP15, and FST}. Some genes identified here have been previously linked to neural tube closure, brain patterning, or NTDs, including \textit{CDX2, HOXB3, IRX3, UTP15, and FST}. Other genes identified have been associated with oxidative and endoplasmic reticulum (ER) stress: \textit{TXNIP, HERPUD1, and CERT1}. For this study, the gene expression profiles of our three VPA samples differed from each other. This prevented the proper analysis of VPA significantly altered GOIs, therefore additional samples have been submitted for RNA-seq analysis.

The small number of GOIs in this study provides a specific and directed approach to future studies to characterize expression profiles in the neural tube, both normal and abnormal, using \textit{in situ} or \textit{immunohistochemistry} to determine whether these genes contribute to the morphological defects resulting from toxicant exposure. Additional studies may examine the role of ER stress as another means by which C2, FB1, or VPA disrupt normal development.
CHAPTER 5: Conclusions

The purposes of this research were four-fold: 1) to characterize the effects of C2-ceramide (C2), fumonisin b1 (FB1), and valproic acid (VPA) on cellular viability, differentiation, and oxidative state; 2) evaluate morphological and histological changes in the developing neural tube of embryos treated with C2, FB1, or VPA; 3) determine whether changes in the redox state of embryonic tissue were altered under treatment conditions, and whether those changes are similar or different across treatments; and 4) evaluate and compare gene expression profiles of embryos treated with C2, FB1, or VPA during neural tube closure.

To address the first specific aim we treated undifferentiated P19 embryonic carcinoma cells with varying concentrations of C2 (6.25-200 µM), FB1 (0.1-800 µM), and VPA (0.05-2.4 mM) to assess viability (MTT assay) and oxidative stress (DCF assay). This informed our working concentrations of 100 µM C2, 100 µM FB1, and 600 µM VPA for all future studies. We then initiated neuronal differentiation in P19 cells and dosed with working concentrations over a 4-day period to assess the effect of each toxicant on cellular differentiation capability. We demonstrated that C2 had the greatest effects on the cell by both inducing ROS and preventing differentiation if exposure occurred within the first 2.5 days of differentiation. We also showed that VPA, while having no effect on ROS production, also reduced cellular capacity to differentiate with early exposure. However, FB1 did not induce ROS or prohibit differentiation in the P19 cells. These results indicate that while all three toxicants have the capability of disrupting neural tube closure, they have unique responses at the cellular level.

In the second specific aim, we exposed chick embryos at the beginning of neural tube closure (HH8) to each toxicant (100 µM C2, 100 µM FB1, or 600 µM VPA) for 24 h and assessed the morphological effects. We demonstrated for the first time that C2 exposure during
neurulation disrupts proper closure, resulting in increased frequency (45%) of NTDs when compared to control (0.5% DMSO). We confirmed that FB1 and VPA exposure increased the incidence of NTDs in the chick embryo (50% and 69%, respectively). We evaluated the types of NTDs, showing that these toxicants replicate human events generating a random number of cranial, caudal, or cranial + caudal defects in affected embryos. Embryos growth was retarded significantly in those exposed to FB1; they were significantly smaller and developed fewer somite pairs over time than untreated embryos. In C2-treated embryos with NTDs, the number of somite pairs was reduced over the 24 h period. Cellular apoptosis was unaffected in C2- and FB1- treated embryos but increased with VPA exposure, while proliferation, measured by evaluating PH3 expression, was reduced across treatments.

For the third specific aim, we measured glutathione (GSH) redox potential (Eh) as an indicator of cellular and tissue redox state. We treated undifferentiated and neuronally differentiated P19 cells to 100 µM C2, 100 µM FB1, or 600 µM VPA and collected samples at various time points (1, 2, 4, 6, 8, 12, or 24 h) to understand how the toxicants were affecting the redox state over time. Undifferentiated cells were unaffected by C2 and revealed minor oxidizing shifts in response to VPA or FB1. However, we showed that differentiated cells were more susceptible to the redox stress of the toxicants. Under VPA exposure, the GSH Eh showed a significantly oxidizing shift in the cellular redox state. C2 induced oxidation after 12 h of exposure, while FB1 had no effect. We similarly exposed chick embryos to each toxicant and collected samples at 1, 2, 4, 8, 12, and 24 h to measure GSH Eh. Three different responses were observed in the embryos: C2 caused an oxidizing shift in the Eh between 1-8 h after exposure, FB1 induced the opposite reaction, resulting in a reducing shift between 1-8 h post exposure, and the GSH Eh showed in initial reducing shift (1 h) followed by an oxidizing shift (4 h) in VPA-
treated embryos. This is the first time three toxicants known to cause the same defect were tested in the same animal model and collected for GSH Eh to compare the resulting shifts in redox state. Because mitochondrial function and oxidative stress are often associated together, we also examined mitochondrial respiration at 12 h post-treatment for embryos exposed to each toxicant. We showed that O2 flux was reduced with C2 and FB1 treatment but not VPA. These data support the concept that an oxidizing or reducing shift in the redox state can lead to protein modifications, resulting in downstream effects that may alter neural tube closure.

Finally, for specific aim four, we compared the gene expression profiles for embryos treated with C2, FB1, or VPA to control embryos 6 h after exposure. We chose 6 h because a shift in the redox state was observed at 1 h in all treatments and resulting changes in gene expression would likely be observed 4-6 hours after these shifts. Our VPA samples all showed very different gene expression profiles, as measured by RNA-seq, therefore comparing them to obtain any significance was impossible. Additional samples are prepped to be sequenced and will be analyzed upon completion. The data obtained from C2- and FB1-treated embryos revealed a concise list of 16 and 20 genes, respectively, that were significantly up- or down-regulated. Of these genes, we identified 10 genes of interest to evaluate in future studies.

In conclusion, this body of work significantly contributes to our understanding of the effects of these NTD-inducing toxicants during early embryonic development. For the first time, C2-ceramide has been reported as a novel inducer of NTDs. The cellular and embryonic GSH Eh profiles were characterized and have shown for the first time that the three NTD-inducing toxicants have differential responses in redox state. This novel comparative analysis combines several descriptive measures to provide evidence of how variable the effects of teratogenic compounds are on the redox state of a developing embryo.
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Gharibi B, Ghuman M, Hughes FJ. 2016. DDIT4 regulates mesenchymal stem cell fate by mediating between HIF1α and mTOR signalling. Scientific Reports 6:36889.


CURRICULUM VITAE

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EDUCATION

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2016-2020 Graduate Research Assistant: BYU, Mentor: Dr. Michael R. Stark
▪ Embryonic ceramide, fumonisin, and valproic acid exposure alters tissue redox balance and increases the incidence of neural tube defects

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▪ Utilization of redox-fluor technology in HeLa cells to measure intracellular response to oxidative stress

2015-2016 Graduate Research Assistant: BYU, Mentor: Dr. Benjamin T. Bikman
▪ Early embryonic ceramide exposure effect on metabolic respiration in chick embryos

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▪ Effects of ceramide exposure on embryonic neurulation and the development of neural tube defects in chick model

2015 EPSCoR Research Intern: UWYO, Mentor: Dr. Donal Skinner
▪ Salt and Fat: Determining the effects of a high salt diet on perirenal adipocyte size

2015 EPSCoR Research Intern: UWYO, Mentor: Dr. Donal Skinner
▪ Salt and Fat: Determining the effects of a high salt diet on lipid storage and metabolism in rats

2014-2015 McNair Scholar Research Intern: UWYO, Mentor: Dr. Donal Skinner
▪ Analyzing the Effects of a High Sodium Diet on the Onset of Puberty and Metabolism of Lipids in Rats
PROFESSIONAL EXPERIENCE

2018-2019  PDBIO 382: Developmental Biology Lecturer and Teaching Assistant
- Prepared and taught 1/3 of all lectures during the semester
- Assisted in assignment and exam preparation and grading
- Conducted individual and group study/review sessions

2017  PDBIO 562: Reproductive Physiology Substitute Lecturer and Teaching Assistant
- Presented lectures on reproductive physiology topics
- Assessed and provided feedback on all student research papers

2016  PDBIO 482: Developmental Biology Teaching Assistant
- Conducted individual and group study/review sessions
- Assisted in assignment and exam grading

2016  PDBIO 363: Physiology Lab Teaching Assistant
- Supervised student lab completion
- Encouraged student learning through lab topic lectures

PUBLICATIONS

Peer-reviewed


In progress


Abstracts

Ross MM, Piorcyznski TB Hansen JM, and MR Stark. A comparative study of developmental toxicants that cause neural tube defects on intracellular redox state. 26th Annual Conference of the Society for Redox Biology and Medicine, Las Vegas, NV Nov 2019, Poster


Ross MM, Hansen JM, and MR Stark. Measuring glutathione redox potential as a tool in understanding the mechanism of failed neural tube closure in response to the developmental toxicants. 10th International Conference on Neural Tube Defects, Austin, TX Oct 2017, Oral


Ross MM, Skinner DC, and D Pitynski. Salt and Metabolism: What’s in the Feces? The 32nd Annual Scientific Meeting of The Obesity Society, Oct 2014, Boston, MA, Poster


**AWARDS**

- 2015-2020 PDBIO Research and Teaching Assistantships
- 2015-2020 PDBIO Tuition Scholarships
- 2016-2019 BYU Research Presentation Awards
- 2019 Young Investigator Award, Society for Redox Biology and Medicine Conference
- 2018 First Place Oral Presentation Award, BYU PDBIO Graduate Student Retreat
- 2017 Second Place Oral Presentation Award, BYU PDBIO Graduate Student Retreat
- 2017 Marcy Speer Memorial Award for Predoctoral Presentation, International Neural Tube Defect Conference
- 2016 First Place Poster Presentation Award, BYU PDBIO Graduate Student Retreat
- 2015 EPSCoR Undergraduate Research Grant
- 2014-2015 McNair Scholar Undergraduate Research Award

**SOCIETY MEMBERSHIPS**

- Society for Developmental Biology
- Society for Redox Biology and Medicine
- Society for Neuroscience
- National Science Teachers Association

**AREAS OF RESEARCH INTEREST**

- Effects of maternal diet on offspring health
- Obesity and development
- Embryonic neurulation and teratogens
- Science education
- Communicating science