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Glyphosate and Anencephaly: Death by A Thousand Cuts

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Abstract

Anencephaly, which occurs when the rostral (head) end of the neural tube fails to close early in embryonic development, represents perhaps the most extreme manifestation of neural tube defects (NTDs). A wide range of developmental events and processes, working singly or in concert, are either known to cause, or are strongly associated with, NTDs in general, and with anencephaly in particular. Glyphosate is the most widely used herbicidal chemical on the planet. Here we review a multitude of ways in which glyphosate can detrimentally impact, or 'cut,' embryological and fetal development to specifically favor the anencephalic phenotype. The evidence presented here includes data gathered from epidemiology, toxicology, general and nutritional biochemistry, and developmental biology. While the case here is often based on statistical associations and plausible biological arguments, we offer clearly defined pathways whereby glyphosate can be seen as likely holding the knife that is inflicting some, or perhaps even most, of these developmental cuts that lead to anencephaly and other NTDs. We offer some suggestions for lines of research to validate or refute our thesis, and conclude with our thoughts on the relevance of this link with regard to public health policy.

Keywords: Anencephaly; Neurodevelopment; Glyphosate; Folate; Methionine; Retinoic acid; Methylation; Gestational Diabetes; Megalin; DHEA Sulfate; Adrenal insufficiency

Introduction

Defects in neural tube closure occur in 1 in 1000 births, making this the second most common birth defect [1]. For the neural tube to close successfully there must be tight coordination in timing between proliferation and differentiation. Complex signaling mechanisms control cellular morphological changes and directed movement to form the tubular shape [2-4]. Anencephaly is one of the most severe forms of neural tube defects (NTDs), reflecting closure failure in the cranial region. It results in the absence of much of the brain, skull, and scalp, and is incompatible with longterm survival. In anencephaly, the brain initially protrudes through a defect in the cranial vault (termed exencephaly), and the brain is then gradually destroyed because of mechanical injury and vascular disruption. Damage to the hypothalamus results in adrenal hypoplasia due to insufficient expression of adrenocorticotrophic hormone (ACTH) by the pituitary gland, which in many cases fails to develop at all.

The causes of anencephaly are multifactorial. While there is believed to be a strong genetic component, the genetic causes are mostly unknown. Certain polymorphisms of the gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) are linked to increased anencephaly risk, and this is likely connected to the observed benefit of folate supplementation to reduce risk. However, estimates suggest that only 13% of neural tube defects can be attributed to mutations in methylene tetrahydrofolate reductase [5]. Genetics almost certainly works together with environment through synergistic effects. For example, the antiepileptic drug valproate shows strong links to anencephaly, likely through its known induction of oxidative stress and disruption of folate homeostasis and histone deacetylase (HDAC) methylation [6].

In this paper, we develop the argument that glyphosate, the active ingredient in the pervasive herbicide Roundup, is a significant risk factor for anencephaly and other neural tube defects. Our exploration of the research literature on anencephaly and on glyphosate's known disruptions of critical vitamins, minerals, metabolic processes and hormonal signaling lead us to the inescapable conclusion that glyphosate disrupts neurodevelopment, leading to teratogenic outcomes, one of which is anencephaly. In the remainder of this paper, we will show how each of the 'thousand cuts' inflicted by glyphosate can help explain the pathophysiology leading to anencephalic pregnancies.

Evidence that Glyphosate Disrupts Neurodevelopment

Industry claims consistently state that glyphosate is a very safe chemical without teratogenic effects, and regulators around the world have used these claims to justify indiscriminate use of glyphosate based formulations. However, a paper published by Antoniou et al. in 2012 was highly critical of this claim, maintaining that even industry sponsored studies demonstrated evidence of harm that was inexcusably ignored, and that independent studies, both in the laboratory and on exposed human populations, have clearly shown otherwise [7]. There is considerable epidemiological and experimental evidence that glyphosate disrupts development, particularly of the cranial region. Studies on chick embryos, tadpoles and zebrafish embryos have shown impaired cranial development at doses well below the typical application rates in agriculture. Multiple epidemiological studies of agricultural populations have shown anomalously high rates of neural tube defects. Compelling arguments can be made that glyphosate is causal in an anencephaly epidemic in Yakima, WA in the last decade.

Glyphosate is an amino acid analogue of glycine, and much of its toxicity is believed to be derived from its disruption of glycine dependent processes. It has been proposed in several recent publications that glyphosate's insidious, cumulative toxicity derives from its ability to insinuate itself into proteins in place of the coding amino acid glycine during protein synthesis [8-11], and that this can explain the long list of debilitating diseases and conditions whose alarming rise in recent decades coincides with the exponential growth of glyphosate use on core crops [12]. The idea that glyphosate can substitute for glycine during protein synthesis remains speculative at this time, although a growing body of evidence supports this idea. There are several naturally produced toxins that derive their toxicity from their ability to displace coding amino acids during protein synthesis [9,10], including the increasingly popular herbicide, glufosinate. There is both epidemiological and biochemical evidence that errorful substitution of glyphosate for glycine has contributed to the rise in several debilitating diseases, including ALS [10], Parkinsonism [8,13] and autism [11]. Furthermore, the bacterial resistance gene that is inserted into GM glyphosate resistant crops involves a genetic mutation that results in a substitution of alanine for the highly conserved glycine at residue 100 in the enzyme's active site [14], preventing glyphosate from disrupting enzyme function by removing that vulnerable glycine residue.

Infants born in agricultural populations where glyphosate-based herbicides are heavily used appear anecdotally to have higher rates of craniofacial malformations. Two studies written in Spanish of populations in Argentina and Paraguay exposed to glyphosate through the extensive cultivation of GM Roundup Ready soy crops both showed obvious evidence of neural tube defects, which were proposed to be due to disrupted retinoic acid signaling [15,16]. Cordoba is an area of intensive planting of GM soy, and it had a higher incidence of spina bifida, mocriota, cleft palate, and postaxial polydactyly than six other regions that were studied [15]. Children born to women exposed to pesticides in Itapua, Paraguay had higher rates of craniofacial defects, anencephaly, microcephaly, hydrocephalus, myelomeningocele, cleft palate, anotia, polydactyly, syndactyly and congenital heart defects [16]. Itapua is also an area where GM soy is intensively cultivated.

A study based in Mexico on agricultural workers found significantly increased risk to anencephaly in a fetus, following exposure for both the father and the mother [17]. Mothers who had worked in agriculture during an acute risk period surrounding the conception date had a 4.57-fold increased risk of producing an anencephalic fetus. The paternal risk was a 2.50 odds ratio during the acute risk period which fell to 2.03 for earlier exposure.

It is impossible to know from these statistics the extent to which glyphosate specifically is the cause. However, a study investigating an association between the risk of neural tube defects and residency within 1000 meters of pesticide application areas in California found an OR of 1.5 specifically for glyphosate [18]. The study period was 1987 to 1991, long before the introduction of GM Roundup-Ready crops and associated dramatic increases in glyphosate usage.

Paganelli et al. systematically studied the effects of glyphosate exposure on chick embryos and tadpoles [19]. Embryos exposed to a 1/5000 dilution of a commercial glyphosate based herbicide or to glyphosate by itself exhibited highly abnormal development, with defective neural crest formation and deformities in the cranial cartilages at the tadpole stage. Similar effects were observed with chicken embryos, showing reduction of the optic vesicles and microcephaly. They suspected that the effect was caused by disruption of retinoic acid signaling, especially since treatment with an RA antagonist rescued the teratogenic effect. This effect could be explained in part by glyphosate's known suppression of cytochrome P450 (CYP) enzymes [20], since CYP enzymes in the liver degrade retinoic acid [21].

A paper published by Roy et al. in 2016 studied the results of low dose glyphosate exposure to zebrafish embryos [22]. They found that glyphosate and glyphosate based formulations induced morphological abnormalities including cephalic and eye reductions in the brain and a loss of delineated brain ventricles. Glyphosate was developmentally toxic to the forebrain and midbrain but not the hindbrain.

A study by Benachour and Séralini showed that glyphosate in various formulations induced apoptosis and necrosis in human umbilical, embryonic and placental cells at dilution levels far below agricultural recommendations and corresponding to residues expected in food or feed [23].

In an experiment by Dallegrave et al. published in 2003, Wistar rat dams were treated with glyphosate at 500, 750 and 1000 mg/kg from day 6 to day 15 of pregnancy [24]. Glyphosate exposure caused a significantly increased risk to skeletal malformations in the fetuses, with 15%, 33%, 42% and 57% of the fetuses affected for the control group and the three increasing glyphosate exposure levels respectively. The most frequent skeletal defects were incomplete skull ossification and enlarged fontanel, which were highly significantly more frequent in the exposed groups ($p<0.001$).

Studies on sea urchin embryos have shown that glyphosate at low exposure levels causes a delay in the cell cycle, which would disrupt early embryonic development [25,26]. It was suggested in [25] that glyphosate and its metabolite aminomethylphosphonic acid (AMPA) alter cell cycle checkpoints by interfering with DNA repair machinery. This could result in cancer but would also disrupt early stages of development.

Between January 2010 and January 2016, 42 babies were confirmed with anencephaly in Yakima, Benton and Franklin counties in Washington State. Over this time period, the rate of anencephaly in these counties was significantly higher than the rate in the general US population. The rates were especially high in 2012 and 2013. The Yakima River serves as the main irrigation source for the Yakima Valley, and it runs through all three counties where anencephaly rates are anomalously high. Beginning in 2009, the Benton County Weed Board received a grant from the DOE to control a noxious invasive weed called flowering rush [26] in the Yakima River basin. It was feared that this weed would hinder recreational use, negatively impact irrigation systems and wetlands, and alter aquatic food webs.

During the next four years, six miles of shoreline were repeatedly treated with glyphosate, which was considered to be the best option due to its perceived nontoxicity. Glyphosate was also used to control other water weeds such as purple and wand Loosestrife and Japanese knotweed. The highest application rates of glyphosate in the Yakima Valley were in the years 2012 and 2013, the same years in which anencephaly rates peaked.

Glyphosate and Nutritional Imbalances

Multiple nutritional deficiencies and imbalances are linked to anencephaly. Glyphosate exposure impairs essential nutrient absorption, utilization, and synthesis. In this section, we briefly review some of the specific affected nutrients, and we will be discussing many of them in greater detail in later sections of this paper. Glyphosate is well established as a chelator of several essential minerals, including zinc, manganese, cobalt, and copper [27-30]. Zinc and cobalt deficiency in particular can be expected to especially impact anencephaly. Glyphosate can also be predicted to deplete the level of multiple vitamins that are linked to anencephaly, including folate, cobalamin and vitamin D. Neural tube development requires refined control over retinoic acid levels which likely gets disrupted through glyphosate's effects on CYP enzymes. Overexpression of retinoic acid is probably the strongest link from the research literature tying glyphosate to anencephaly. A brief summary of glyphosate's adverse effects on certain nutrients critical for neural tube closure is provided in table 1.

Table 1: Summary of effects of glyphosate on various nutrients known to be critical for proper neural tube closure. See text for details.

Nutrient	Mechanism
Zinc	Chelation by glyphosate
Cobalamin	Inhibition of ALA synthesis, cobalt chelation
Folate	Product of Shikimate pathway which glyphosate inhibits
Retinoic Acid	Disrupted metabolism by CYP enzymes
Methionine	Impaired synthesis from inorganic sulfur by *E. coli* and plants
Vitamin D	Disrupted activation by CYP enzymes

Zinc deficiency

Low maternal serum zinc levels have been strongly linked to anencephaly and other congenital malformations [31-35]. Glyphosate is a well established strong mineral chelator. In fact, it was first patented as a pipe cleaner for this reason [36]. Among all essential minerals, the highest stability constants are found in the glyphosate-copper complex and the glyphosate-zinc complex [27,28].

Glyphosate's toxic effects have been directly linked to plasma zinc deficiency caused by sequestration of zinc into metallothionein (MT) following oxidative stress. A study from 2013 found that subchronic glyphosate exposure to Wistar rats induced histopathological damage in multiple organs [37]. Zinc supplementation prior to glyphosate exposure ameliorated these effects. MT activation results in the depletion of zinc from other zinc dependent proteins, and zinc will accumulate in the liver, bound to MT, at the expense of serum levels of zinc, leading to systemic zinc deficiency [38]. Interestingly, the well established link between maternal valproic acid exposure and neural tube defects is also attributed to MT sequestration and subsequent plasma zinc deficiency [39]. Indeed, it has been demonstrated in multiple studies that MT is activated by many different stressors, especially toxic metals such as cadmium [40] but also valproic acid [39], polyphenols [41] and lipopolysaccharides [42].

MT is a small, cysteine rich protein which binds to heavy metals and consumes free radicals. Its protection from oxidation damage surpasses that of glutathione [43]. The rate constant for reactions of hydroxyl radicals with MT is about 340-fold higher than that with glutathione [44]. MT crucially depends on zinc for its proper function. Glyphosate may also disrupt MT's function by substitution for critical glycine residues. The amino acid sequence of all four variants of the MT protein contains at least one, and sometimes more, highly conserved glycine residues [45]. For example, the N-terminal domain of Type 2 MTs is highly conserved as the sequence MSCCGGNCGCS, with three glycine residues [45].

MT is induced by various cytokines expressed in the acute phase response, including TNF-α, IL-1, and IL-6. Glyphosate has been shown to induce TNF-α expression in hepatocytes of sub-lethally exposed rats [46]. MT expression was also significantly increased in a study by Mottier et al. on oysters exposed to glyphosate [47]. Glyphosate exposure has been linked to oxidative stress in multiple organs [48,49], and impaired MT function would enhance the damaging effects.

Epidemiological studies strongly support a role for zinc deficiency in anencephaly. A study based in Iran comparing 23 mothers of neonates with neural tube defects and 36 control mothers showed that significantly more of the case mothers (56.5%) had zinc deficiency than the controls (19.4%) [32]. A study investigating serum mineral levels in women with second trimester induced abortion following a diagnosis of neural tube defects revealed that zinc levels were anomalously low ($p<0.001$), whereas copper and lead levels were elevated [31]. In a case study, a female who had twice previously given birth to anencephalic infants was supplemented with zinc sulfate prior to her third conception and throughout her pregnancy. At term she gave birth to a healthy baby boy [50].

Vitamin B12

Reduced serum B12 (cobalamin) has been linked to anencephaly for almost 40 years. Glyphosate can plausibly cause deficiency of this nutrient *via* both enzymatic inhibition and chelation of the cobalt ion.

Deficiency of vitamin B12 was first proposed to be linked to NTDs and specifically to anencephaly in a letter published in The Lancet in 1980 [51]. Surprisingly, Suarez et al. found that the lowest quartile of serum vitamin B12 conferred an OR of 3.0 for NTDs, which was significantly higher than the OR for serum folate or RBC folate [52]. More recently, Molloy et al. confirmed the excessive number of anencephalic births among women entering pregnancy with the lowest serum B12 levels, a risk that was again found to be independent of RBC and serum folate [53].

It has been shown that in plants, glyphosate inhibits the production of δ-aminolevulinic acid (ALA), likely through inhibition of ALA dehydratase (ALA-D), the rate-limiting initial step in the synthesis of chlorophyll and other porphyrin ring compounds [54]. ALA-D activity has been shown to be significantly reduced in farm workers exposed to glyphosate [55]. Biosynthesis of ALA is the first step in the production of both vitamin B12 and heme in animals, due to its central role in the building of the pyrrole rings common to both molecules [56]. There are no studies that have looked specifically at depletion of vitamin B12 by glyphosate, but its documented interference with pyrrole synthesis, as well as its depletion of probiotic bacteria that synthesize the vitamin, make disruption of its supply highly probable.

Another cut induced by glyphosate has to do with its chelation of cobalt, the elemental anchor for the tetrapyrrolic structure of cobalamin. Cobalt has been shown to be directly chelated by glyphosate [57]. In a European study of cattle exposed to and excreting glyphosate in their urine, cobalt was virtually undetectable in the serum of the exposed cows, more significantly depleted than any other tested mineral [58].

Folate

Compelling evidence supports the idea that folate plays a critical role in neurodevelopment. In fact, concerns over increases in neural tube defects led to regulation in the United States to require folic acid fortification of wheat based products starting in 1998, based on the premise that this would reduce the incidence of neural tube defects. Surprisingly, studies by the Centers for Disease Control monitoring serum folate levels in women of reproductive age found that the mean folate levels actually decreased during the observation interval (1999-2004) following the introduction of fortified wheat [59]. This was consistent across race (Caucasians, Blacks and Hispanics) with the level in Caucasians falling from 13.4 in 1999-2000 to 12.1 in 2001-2002 to 11.3 in 2003-2004.

Genetically engineered Roundup-Ready crops were ramping up dramatically during that same time interval. This suggests that glyphosate exposure from contaminated food may have cancelled the benefits of folic acid enriched wheat. Human cells are unable to synthesize folate, and we depend upon our gut microbes to provide us with adequate folate. Folate is derived from chorismate, a product of the shikimate pathway, which is the pathway that is disrupted by glyphosate in plants, as a key factor in glyphosate's toxicity to plants. Supplementation of folic acid has been documented to result in approximately a 60% reduction in the risk of NTDs in the United States [60]. However, these numbers are hard to interpret in the face of increasing use of prenatal screening activities. In 1985, Lorber and Ward expressed optimism that spina bifida might soon nearly completely disappear as a congenital defect due to the increasing use of antenatal diagnostic facilities that could facilitate early detection

followed by elective abortion [61]. Since then, such diagnostic tools have been widely used among the industrialized nations, complicating the picture with respect to monitoring rates of anencephaly over time.

There is not universal consensus about the mechanism by which folic acid prevents NTDs [62], although evidence supports an important role for improved methylation capacity. The decline in anencephaly since mandatory folic acid fortification of food in the US has been less dramatic than the decline in spina bifida (31% decline for spina bifida *vs* 16% for anencephaly), but this still suggests that folate has a direct role to play [63].

Glyphosate's herbicidal action is due, at least in part, to its inhibition of the shikimate pathway [64,65], which is active in plants and bacteria, but does not exist in animals [66]. However, both *Lactobacillus plantarum* and several species of *Bifidobacterium*, important species in the human microbiome, have been shown to biosynthesize folate. A probiotic formulation of folate producing *Bifidobacteria* resulted in an increased folate level in the serum of rats [67]. A human trial of the same supplement raised folate levels in the feces. Glyphosate's inhibition of the shikimate pathway will reduce the amount these bacteria can contribute to maternal serum folate. Furthermore, a study on the poultry gut microbiome showed that *Bifidobacteria* and *Lactobacillus* species were especially susceptible to glyphosate toxicity [68].

Retinoic acid

The teratogenic activity of retinoic acid (RA) was first described in 1967. In that experimental report, only one rat out of 19 whose mothers were fed 5 mg RA daily for 9-11 gestational days was born live. This rat had anencephaly as well as cleft palate [69]. Since that time a great deal has been discovered about the role of RA in both normal embryonic development and in its contribution to congenital defects. Important in this regard is that embryonic RA levels are tightly controlled through both synthesizing and degrading enzyme activity, thus establishing RA gradients within the embryo that regulate many aspects of the developmental process [70].

Glyphosate directly enhances the effects of RA, and exposure to glyphosate during embryogenesis leads to teratogenic effects specifically through its impact on RA signaling. These effects include microcephaly, holoprosencephaly (failure of the brain to develop into hemispheres), and cebocephaly (failure of the nose to fully develop) [19]. Glyphosate's impact on CYP enzymes in the liver that degrade RA [71] could lead to accumulation of RA in exposed mothers, disrupting embryonic fine tuned timing control and adding insult to the injury of enhanced RA signaling activity just mentioned. Exogenous retinoic acid can induce both anterior (anencephaly, exencephaly) and posterior (spina bifida) neural tube defects depending on the developmental stage of treatment [72]. A CYP enzyme, mP450RAI, catabolizes retinoic acid, and it was found to be abundant in retinoid poor regions of the caudal embryo. It is induced by retinoic acid treatment *in vivo*, and this suggests that it serves to tightly regulate retinoic acid levels during development. It is well established that glyphosate inhibits CYP activity in the liver [20,71].

We are unaware of studies that have examined serum RA levels during the first trimester in women giving birth to anencephalic children, but we believe that future studies of anencephaly should include this measurement in order to confirm or refute its potential role.

Methionine

Folate is but one of many factors controlling methylation capacity. More generally, the larger metabolic issue of methylation pathways looms large in impairments in neural tube closure [73]. In addition to disrupting folate synthesis, glyphosate also causes a deficiency in methionine, which has been shown to be essential for neural tube closure in *in vitro* studies. A study by Coelho and Klein examined neural tube closure of rat embryos cultured on cow serum with and without methionine supplementation [74]. Without methionine, the neural tube still fused, but failed to close because the tips of the neural folds failed to turn in. It appears that this step requires methylated amino acids in embryonic neural tube proteins, which fail to form in the absence of methionine.

Human cells are unable to synthesize methionine from inorganic sulfate, so we depend on our gut microbes and diet to supply methionine. A study on carrot cell lines revealed deficiencies in methionine as well as the aromatic amino acids upon exposure to glyphosate [75]. Glyphosate suppressed multiple enzymes involved in the assimilation of inorganic sulfate into methionine in *Escherichia coli* [76]. Methionine is essential for the supply of methyl groups in the methylation pathway, and impaired methylation, associated with folate deficiency, has strong links to impaired neural tube closure and anencephaly [77].

Vitamin D

Vitamin D deficiency has become a world-wide problem in the past two decades, in step with the increased use of glyphosate on core crops [78-80]. Vitamin D is activated to $25(OH)D_3$ in the liver by liver CYP enzymes. Glyphosate has been shown to suppress liver CYP enzyme activity [20], and this can be predicted to induce a deficiency in vitamin D through impaired activation.

A study on mice published in 2015 showed that vitamin D supplements could completely prevent neural tube defects induced through exposure to lipopolysaccharides (LPS) [81]. Dams exposed during gestational days 8-12 produced litters where one in four of the fetuses suffered from neural tube defects. Those dams exposed with equal amounts of LPS but also supplemented with vitamin D produced litters that were completely free of neural tube defects. Vitamin D was shown to significantly reduce inflammation in the placenta and also promoted folate uptake by the placenta.

Metabolic Disorders

In this section, we describe multiple ways in which glyphosate can be expected to interfere with metabolic processes important to neurodevelopment. Throughout, we focus on the idea of glyphosate substitution for glycine in critical proteins and expected consequences. We demonstrate, where available, evidence from the research literature of developmental impairments induced by glyphosate that support the hypothesis of substitution for glycine as a pathological mechanism. In preparation, a brief summary of several proteins known to have highly conserved glycine residues that are essential for their function and the expected consequences of glyphosate substitution are summarized in table 2.

Diabetes

Diabetes, Anencephaly and Glyphosate: Swanson et al. found a very strong correlation between the rise in diabetes incidence and the rise in the use of glyphosate on core crops over the past two decades in the United States [12]. Samsel and Seneff (2016)) [8] discussed a mechanism for glyphosate to cause type II diabetes, based on substitution for essential glycines in the insulin receptor. The insulin receptor contains at least 8 repeats of a highly conserved glycine centered motif [82] that is essential for transport to the plasma membrane [83].

The correlation between congenital defects and maternal diabetes has been noted for nearly 50 years [84], and the more specific link between maternal hyperglycemia and NTDs was established by Reece et al. in 1985 [85]. Subsequent studies have confirmed and extended this association to encompass obesity as well [86,87]. The causal relationship between obesity and NTDs is not yet fully established, but hyperinsulinemia likely plays a role [88]. Ray et al. found the risk of NTDs during fetal development to be greater than 4 times higher in obese women who reported the

Table 2: Various proteins with highly conserved glycines whose substitution by glyphosate would disrupt protein function leading to neurodevelopmental disorders. Associated references are provided. See text for details.

Protein	Conserved G	Effect of glycine substitution
Insulin receptor	G-centered motif	Impaired membrane transport; diabetes [82]
Folate receptor	G137	Impaired folate binding [138]
LDL receptor	G34	Fetal DHEA-sulfate deficiency [140]
CDK1	GEGTYG motif	Cell cycle delay; inhibited mitosis [152]
Kinases	GxGxxG	Enhanced activity; impaired timing in development [158]
Tyrosine phosphatase	G127	Apoptosis in brain and spinal cord [157]
ACTH	Terminal glycine	Impaired activation [181]
Metallothionein	MSCCGGNCGCS motif	Impaired antioxidant capacity [45]
Serine protease	G193	Impaired neural tube closure [126]
Myosin	G699	Holoprosencephaly; Impaired folate uptake [121]

highest dietary intake of high glycemic index foods prior to gestation. Interestingly, this risk was independent of diabetes and of folic acid supplementation [89]. In a similar study, Yazdy et al. found that a high glycemic index diet and high glycemic load diet led to a 50% and 80% increased risk of NTDs, respectively [90]. Over the past two decades most studies and reviews of this topic have confirmed the strong relationship between hyperglycemia and NTDs in *in vitro* [85], animal [91,92], and human studies [93]. Pregestational diabetes has also been repeatedly shown to be a significant risk for NTDs either independently [94,95], or in conjunction with obesity [96]. This correlation is found in a majority, but not all studies [97].

It is well established that hyperglycemia induces a state of elevated oxidative stress and related oxidative damage in many tissues throughout the body [98,99]. The nervous system is particularly susceptible to such damage [100-102]. With respect to NTDs in particular, it has been proposed that the increased oxidant load results in differential gene expression in the developing embryo. For example, in an investigation on diabetic mice, Phelan et al. discovered that NTDs were the result of suppression of the Pax-3 gene, which is essential for neural tube closure [103]. Chang et al. confirmed this and linked Pax-3 suppression directly to oxidative stress induced through depletion of embryonic glutathione [104]. This same embryonic suppression of Pax-3 due to maternal diabetes has been confirmed in at least 3 other studies [105-107].

S100β, iNOS and Gestational Diabetes: A study involving 40 patients who were poisoned by either glyphosate (23 patients) or glufosinate (17 patients) revealed a correlation between serum levels of S100β and neurological symptoms [108]. The difference in serum levels between patients with altered consciousness and seizures and patients not experiencing these symptoms was highly significant ($p<0.001$).

S100β is an acidic calcium-binding protein that regulates several cellular functions, including cell growth, contraction and intracellular signal transduction. S100 protein is elevated in the amniotic fluid of anencephalic fetuses [109,110], likely due to overexpression in the pituitary gland [111]. Serum levels of S100β were significantly higher in fetuses with neural tube defects (2.71 μg/L) compared to controls (0.98 μg/L) [112].

High levels of S100β result in a potent induction of inducible nitric oxide synthase (iNOS) activity in astrocytes [113]. Genetic studies have revealed that genetic variants in all three isoforms of NOS are implicated in neural tube defects, both independently and through interactions with MTHFR variants [114]. Nitric oxide plays an important role during neural tube development in regulating the balance between cell cycle progression and cell death [115].

One likely mechanism by which maternal gestational diabetes increases the risk of NTDs is through increased expression of iNOS. iNOS levels are elevated in embryos of diabetic mice. A remarkable study on mice with streptozotocin-induced diabetes showed that an iNOS inhibitor was able to significantly reduce the incidence of neural tube defects in offspring [116]. It specifically prevented apoptosis in the head region of fetuses, indicating that iNOS is involved in diabetes-related congenital malformations. Further confirmation came from the observation that diabetic iNOS-/-mice produced no fetuses with NTDs. Increased iNOS activity during organogenesis is likely a major factor in the pathogenesis of diabetes-induced malformations.

Methionine Deficiency and Impaired Myosin Function

The importance of cytoskeletal proteins in cell morphology and particularly in neural tube closure is well established. An *in vitro* study on rat embryos demonstrated that, without methionine supplementation, the neural tube failed to close, and the effect was thought to be due to impaired microfilament contraction [74]. A reduction in microfilament associated methylated amino acids in the embryonic neural tube proteins supported this hypothesis. More specifically, levels of dimethylarginine and 30-methylhistidine are reduced in embryos cultured in methionine deficient medium [74]. Actin and α-tubulin, important proteins in the cytoskeletal microfilaments, have been shown specifically to become methylated during neural tube development, and methionine deficiency led to their failure to migrate to the basal cytoplasmic locale in developing neurons [117].

Myosin impairment may also be a factor in defective neural tube closure. Cytoskeletal myosin II complexes are molecular motors that can both bind to and cross link actin filaments into higher order structures. They also translate chemical energy into force production inside the cell by coupling ATP hydrolysis to movement along an actin filament [118,119]. Myosin IIB is required for the morphogenetic movements that drive neural tube closure in Xenopus [120]. Defects in the cell shape and in cortical actin cytoskeletal integrity associated with depleted myosin IIB during neural convergent extension are consistent with reduced cortical tension and altered biomechanical properties.

E. Kinose et al. (1996) [121] devised a set of elegant experiments involving substitution of alanine for an essential G699 residue in myosin to demonstrate the essential role that this residue plays in myosin contractive strength. Alanine substitution reduced its contractile strength to only 1% of the normal capacity. It can be anticipated that glyphosate substitution for this essential glycine residue would have an even more drastic effect, given its bulkier size and negative charge.

Trypsin and Serine Proteases

If glyphosate can substitute for glycine during protein synthesis, there are few areas in which this would cut more deeply than within the serine protease trypsin. Trypsin is produced in the pancreas initially as an inactive

proenzyme. Its activation is a 3-step process. The first two happen within the acinar cells of the pancreas, where methionine and a transport protein are cleaved off within the endoplasmic reticulum [122]. As this modified trypsinogen enters the lumen of the duodenum, duodenal epithelial cells begin secreting enteropeptidase, which hydrolyses a leucine-isoleucine bond, allowing the inactive trypsinogen to swing its 4 polypeptide stretches that make up its activation domain into their more compact and markedly less flexible configuration necessary for enzyme function [123]. Prior to this hydrolysis, the arms of the activation domain have a great degree of flexibility, with a high degree of uncertainty regarding the position of its atoms. Strikingly, trypsin's activation domain contains 4 crucial glycine rich subdomains: N-terminus to Gly 19, Gly 142 to Pro 152, Gly 184 to Gly 193, and Gly 216 to Asn 223 [124].

Gombos et al. studied these glycine 'hinges' in more detail. It was found that, when these hinge glycines are substituted with alanine, the resulting "activated" enzyme retained proenzyme features and lost its proteolytic capacity to varying degrees, depending on the location of that substitution [125]. Utilizing 3 different assay methods, Samsel and Seneff reported that purified trypsin, upon dissociation, had been found to contain radiolabeled glyphosate to a concentration of 62 ppb [9] strongly suggesting that glyphosate is substituting for at least some of these glycine residues.

If the compromised trypsin activity were to only impact the digestive process, that would be concerning enough. But the cut of glyphosate runs much deeper. Schmidt et al. demonstrated that substitution of the highly conserved glycine residue common to trypsin and virtually all serine proteases results in a dramatic reduction of enzyme function [126]. Trypsin is produced by the fetal pancreas as early as week 16-18 [127]. Given the lack of need for digestive enzymes on the part of the fetus, this might seem peculiar. However, in the past two decades the role of a whole class of serine proteases in fetal development has been uncovered. In 2010, Camerer et al. [128] reported an unexpected role for serine proteases in the closure of the neural tube. The activation of two protease activated receptors, PAR-1 and PAR-2, was required for normal development and complete closure of the neural tube.

These proteases are anchored in the membrane of the developing ectoderm. Knockout of PAR-1 and PAR-2 in mice results in complete failure of neural tube closure [128]. Serine proteases are integral to the embryonic and fetal developmental process. Additional roles for these membrane-bound serine proteases are reviewed by Szabo [129].

Legge and Potter found that fetal trypsin production in anencephalic pregnancies was significantly less than in normal pregnancies. Of all other assessed abnormal pregnancy types, including spina bifida, only Trisomy 21 achieved statistical significance in this respect [130]. This suggests that reduced trypsin activity, whether *via* reduced amount or by reduced activity resulting from glyphosate interference, could contribute to anencephaly.

Homocysteine Thiolactone

It has been challenging to determine exactly how folic acid deficiency leads to neural tube defects. However, a growing body of literature supports the hypothesis that the key factor disrupting neural tube development is excessive exposure to homocysteine and homocysteine thiolactone [131-133]. At pathological concentrations, homocysteine induces iNOS expression in macrophages, which results in nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activation [134]. NF-κB plays essential and complex roles in neurogenesis [135], and it can be expected that overactivation would induce pathological consequences.

Folate depletion leads to homocysteine accumulation because folate is a cofactor in the synthesis of methionine from homocysteine. In a study conducted on 103 cases of women with NTD affected pregnancies compared to 139 controls, it was observed that elevated homocysteine levels increased the odds ratio for NTDs, regardless of whether folate or B12 was deficient [132]. When methionine is deficient, there is a danger that homocysteine will erroneously substitute for methionine during protein synthesis. An error editing reaction, catalyzed by methionyl tRNA synthetase, prevents incorporation of homocysteine into tRNA and subsequently into the synthesized protein, by converting it to homocysteine thiolactone [136]. Hence, homocysteine thiolactone accumulates under conditions of methionine deficiency.

A study involving avian embryos demonstrated that both homocysteine and homocysteine thiolactone exposure led to neural tube defects [131]. Specifically, 200 mM D,L-homocysteine or 100 mM L-homocysteine thiolactone during the first three days of embryonic development resulted in neural tube defects in 27% of the embryos. Defective development of the heart and ventral wall were also observed. Folate supplementation greatly suppressed the levels of serum homocysteine and prevented the teratogenic effects.

It was proposed by Denny et al. that a possible mechanism by which homocysteine thiolactone might disrupt neural tube development is through post-translational modification *via* "homocysteinylation" of lysine and/or cysteine residues in the folate receptor [137]. Such homocysteinylation leads to the production of auto-antibodies to the protein and subsequent pathology. It is also possible that glyphosate can cause autoantibodies to the folate receptor as well as receptor dysfunction through substitution for the glycine residue, G137, which is a highly conserved residue at the site of folate binding [138].

Homocysteine acts as an N-methyl-D-aspartate (NMDA) receptor antagonist, and this is also a means by which it could disrupt neural tube closure [133]. Glycine and other glycine site agonists such as D-cycloserine were able to suppress the effects of homocysteine, with glycine itself being most effective at reducing defects ($P<0.001$). Glyphosate, through its suppression of methionine synthesis and folate synthesis by gut microbes, can be expected to induce a state of hyperhomocysteinemia which would lead to impaired neural tube closure. In another cut, glyphosate, as a glycine mimetic, may also disrupt glycine signaling directly by interfering with glycine binding to the receptor.

LDL Receptors and Megalin

LDL receptors (LDLR), expressed on the cellular plasma membrane, mediate the endocytic uptake of LDL by hepatocytes and other epithelial cells. Genetic defects in the LDLR pathway lead to familial hypercholesterolemia [139]. In order to remove LDL from plasma, the receptors must be located in a polarized fashion at the hepatocyte sinusoidal surface. A genetic mutation involving a substitution of aspartic acid for a critical glycine 34 residue within the cytoplasmic tail domain of the LDL receptor, commonly found in the Finnish population, is associated with familial hypercholesterolemia [140]. This glycine residue appears to be essential for polarized targeting of the receptor to the basolateral membrane, and it underlies the metabolic abnormality resulting in reduced liver LDL clearance *in vivo*. Remarkably, substitution of aspartic acid for glycine effectively reverses the polarity of the LDLR, causing it to be mistargeted to the apical surface rather than the basolateral surface, and thus preventing it from functioning in its normal role to endocytose LDL particles. Glyphosate substitution for this critical glycine would also be severely disruptive.

Low density lipoprotein receptor related protein 2 (LRP2), also known as megalin, is highly expressed in the proximal renal tubules, where it likely is involved in LDL endocytosis [141]. Intriguingly, myosin, assisted by the protein Disabled-2, is critically involved in the endocytic process of megalin in proximal renal tubules [142]. We have already described how myosin is severely disabled when glycine residue 699 is displaced.

Defective endocytosis of LDL cholesterol due to glyphosate exposure may be a factor in the severe kidney disease that agricultural workers in Sri Lanka and in the sugar cane fields of Central America are experiencing [143]. This disease affects specifically the proximal tubules.

Myosin is highly expressed in the neural crest during neural tube folding, and likely plays a similar role there as well. Megalin is also expressed in the neuroepithelium of the early embryo, and its absence in knockout mice results in holoprosencephaly, where the forebrain fails to develop into two hemispheres [144]. Such a defect usually results in fetal death. Thus, defective myosin due to glyphosate substitution for G699 can be expected to cut like a knife and lead to failed endocytosis of cholesterol, resulting in defects in neural tube development.

Megalin deficiency in mice leads to loss of cobalamin through failed renal reabsorption [145], and megalin is also essential for the uptake of folate both into kidney proximal tubules and into the neuroepithelium of the developing embryo [146]. In fact, megalin appears to present the main uptake pathway for folate in the neural tube. Furthermore, LRP2 mediates the uptake of many hormones and vitamins bound to their carrier proteins, including vitamin D, retinol, and sex hormones.

Disruption of Kinases and Phosphatases

Neural tube closure requires well choreographed shaping and folding of the neural plate and subsequent midline fusion. This is accomplished through tight spatiotemporal regulation, leading to exquisite timing of cell division, differentiation and morphogenetic movement [147]. An imbalance in the mechanical forces and/or the timing of proliferation and differentiation can both lead to failure, with catastrophic effects.

A critical component of successful morphogenetic cell shape changes is the ability to synchronize the arrest of the cell cycle by stalling the cycle in the G2 phase, which is mediated through the actions of threonine/serine kinases such as cyclin dependent kinase 1 (CDK1) [148,149]. Activation of CDK1 is necessary to trigger entry into mitosis and a commitment to further cell division. CDK1 is a 34-kDa proline directed serine/threonine protein kinase [150], and its activation depends in part on threonine phosphorylation by a CDK activating kinase (CAK). Various so called Weel family kinases phosphorylate CDK1 on a tyrosine reside (Y15) as well as on the adjacent threonine residue (T14) to inactivate the protein. Thus, both activation and inactivation depend on functional protein kinases. Once activated, CDK1 catalyzes the phosphorylation of hundreds of different substrates at a site matching a S/TPx(x)R/K motif.

A study by Coullery et al. in 2015 demonstrated that glyphosate causes irreversible abnormal growth and delayed development in neuronal cells taken from embryonic rats [151]. Sublethal exposure levels of glyphosate led to pathological changes in neurons including a delay in differentiation, shorter and unbranched axons and less complex dendritic branching. These authors identified downregulated Ca^{+2}/calmodulin dependent protein kinase II (CaMKII) activity as a mechanism by which glyphosate caused this pathology. CaMKII is normally inactivated through phosphorylation of a serine-26 residue within the sequence, LGKGAFSVV. Serine phosphorylation introduces a negative charge in the vicinity that interferes with ATP binding.

A study by Samsel and Seneff [8] argued that glyphosate substitution for one of the nearby glycines might be expected to also inhibit ATP binding through the introduction of negative charge. A similar phenomenon can be predicted to lead to inhibition of CDK1, and, in fact, the argument is even more compelling in this case because the tyrosine/threonine pair that are phosphorylated to inactivate the enzyme are couched between two highly conserved glycine residues, with the conserved motif GEGTYG [152].

A study from 2004 on glyphosate's effects on cell cycle regulation in zebrafish is consistent with the idea that glyphosate suppresses the activity of CDK1 [153]. These authors studied the effects of various glyphosate based formulations on the first cell division of sea urchin embryos, a recognized model for cell cycle studies. They found that all formulations, at low concentrations, caused a delay in the first cell cycle, and, in some cases, even complete inhibition of mitosis. They identified disruption of the CDK1/cyclin B complex as the key toxic effect. They noted that the concentrations typically used in agriculture are 500 to 4000 times higher than the threshold adverse concentration towards the cell, and suggested that this effect would be expected to increase cancer risk in exposed agricultural workers. This conclusion is supported by studies showing increased risk to cancer among agricultural workers exposed to glyphosate [154]. It seems to us that it could also contribute to the increased risk to neural tube defects among offspring of pesticide applicators, particularly females [17].

Protein kinase phosphatases remove phosphates from proteins, often resulting in inactivation. A calmodulin dependent protein kinase phosphatase is indispensible for normal embryogenesis in zebrafish [155,156]. Knockout of this phosphatase leads to significant morphological abnormalities resulting in a number of apoptotic cells in the brain and spinal cord of the abnormal embryos. Substitution of proline or alanine for the conserved G127 residue in tyrosine phosphatase resulted in a 400-fold decrease in catalytic activity [157]. On the other hand, glyphosate substitution for critical glycines in a specific glycine-rich loop motif in kinases would be predicted to increase their expression [8]. The result would be systemic hyperphosphorylation, disrupting the timing of neuronal development. Protein kinases have a highly conserved glycine rich loop (GXGXXG) [158] which, according to modeling, forms an elbow around the nucleotide [159]. The second glycine, G48, is conserved in 99% of protein kinases. Its replacement by a negatively charged residue produces mutants that exhibit faster release of ADP from the ATP pocket, resulting in increased activity. Glyphosate substitution can be expected to do the same, according to basic biophysical principles.

Thus, if glyphosate can erroneously substitute for glycine during protein synthesis, it can be predicted to have devastating and unpredictable consequences to both kinases and phosphatases, disrupting the delicate balancing act that orchestrates the complex timing and sequencing of neural tube closure events.

DNA Damage and Micronucleus Formation

Children with neural tube defects show significant DNA damage in blood comet assays compared to controls. A study from 2012 showed that severe cases of NTDs like anencephaly and meningomyelocele had an increased percentage of DNA damaged cells compared to less severe cases such as spina bifida [160].

During the process of mitosis, if a chromosome does not appropriately attach to the mitotic spindle, it can be left behind as a "micronucleus." The number of such micronuclei in polychromatic erythrocytes can serve as an indicator of mitotic checkpoint failure and cell cycle arrest [161]. An overabundance of micronuclei is a sign of genotoxic events and chromosomal instability. The micronucleus test is recognized as one of the most inexpensive, reliable and convenient assays for genotoxic carcinogens. The antiestrogen tamoxifen and the DNA damaging drug cisplatin induce cell-cycle arrest and micronucleus formation in breast cancer cells [162]. Some tri- and tetra-chlorobiphenyls (PCBs) have also been shown to induce both micronuclei and cell cycle arrest in various mammalian cell lines [163].

Glyphosate has been shown to induce DNA damage in fish erythrocytes at extremely low doses (parts per billion) [164]. Several studies have demonstrated that glyphosate exposure induces increased micronucleus formation in various species [165-167]. A meta-analysis combining

81 experiments specifically assessing micronucleus frequency in cells exposed to glyphosate established with high statistical significance that glyphosate causes micronucleus formation [168]. The studies involved multiple species, including mice, fish, alligators, amphibians, and onion. Roundup and other formulations were found to cause micronucleus formation at lower glyphosate exposure levels than glyphosate alone.

Glyphosate can also be expected to induce micronucleus formation indirectly through its effects on homocysteine and retinoic acid, as discussed previously. Both of these are linked to DNA damage and excessive micronucleus formation [169,170].

Disrupted Fetal Development

In this section, we review the pathologies associated with the intrauterine development of the anencephalic fetus, including a defective hormonal system and placental stress, and we discuss how glyphosate could play a role in these pathologies. Figure 1 schematizes the disrupted hormonal pathways that lead to underdevelopment of the fetal brain in anencephaly.

Pituitary and Adrenal Glands

Normal fetal development is critically dependent upon both the maternal and fetal hypothalamus pituitary adrenal (HPA) axis. While the fetus produces a limited amount of ACTH starting in week 12 of development, most circulating fetal ACTH originates from the placenta, which produces both ACTH and corticotropin releasing hormone (CRH) [171]. ACTH stimulates the development and hypertrophy of cells within the fetal adrenal zone [172], while ACTH and CRH of both fetal and placental origin stimulate fetal adrenal cortical cells to produce the androgen DHEA-S [173]. By mid gestation, the fetal zone occupies up to 90% of the total fetal adrenal gland, and is producing 100-200 mg/day of DHEA-S [174]. At term, the fetal adrenal has grown to nearly the same size as the fetal kidney, and is producing more DHEA-S than the adult adrenal gland produces [175].

High DHEA-S production by the fetus is dependent upon a) continuous stimulation by CRH that originates in the placenta [176]; b) adequate amounts of cholesterol esters, carried predominantly as LDL particles; and c) LDL receptor number and integrity to allow for LDL's binding and delivery of the cholesterol to the cell interior. This sequence of events is described in detail in the fantastic review by Rainey *et al.* previously referenced [175]. Also, it is critical to note that development of the anencephalic phenotype is closely related to both the maternal and fetal hypothalamus pituitary adrenal (HPA) axis and the altered production of hormones therein.

While the fetus produces a limited amount of ACTH starting in week 12 of development, most ACTH in fetal circulation originates in the placenta and possibly the fetal membrane [177], which produces both ACTH and CRH [171]. Maternal pituitary ACTH likely contributes little to circulating fetal ACTH [178], so the majority impacting fetal development must come from placental origin. Begeot et al. demonstrated that, in the anencephalic fetus, lack of hypothalamic development results in significantly reduced or absent fetal CRH. This in turn leads to diminished pituitary development and subsequent reduced production of several pituitary hormones, including ACTH [179]. Fetal growth and development are critically dependent upon production of ACTH, without which the fetal adrenal gland fails to properly hypertrophy.

In the pituitary, ACTH is synthesized *via* enzymatic cleavage of a precursor molecule [180]. This involves the activity of a copper dependent enzyme to carry out alpha amidation of an extended glycine residue on the ACTH precursor [181]. In fact, ACTH is one of several signaling peptides whereby a terminal glycine of a prohormone is acted upon enzymatically to create the bioactive peptide with an alpha amide [182]. This process is so reliant upon the terminal glycine across many classes of propeptides that the names of two enzymes carrying out the process, both ubiquitous throughout the animal kingdom, reflect glycine's role in

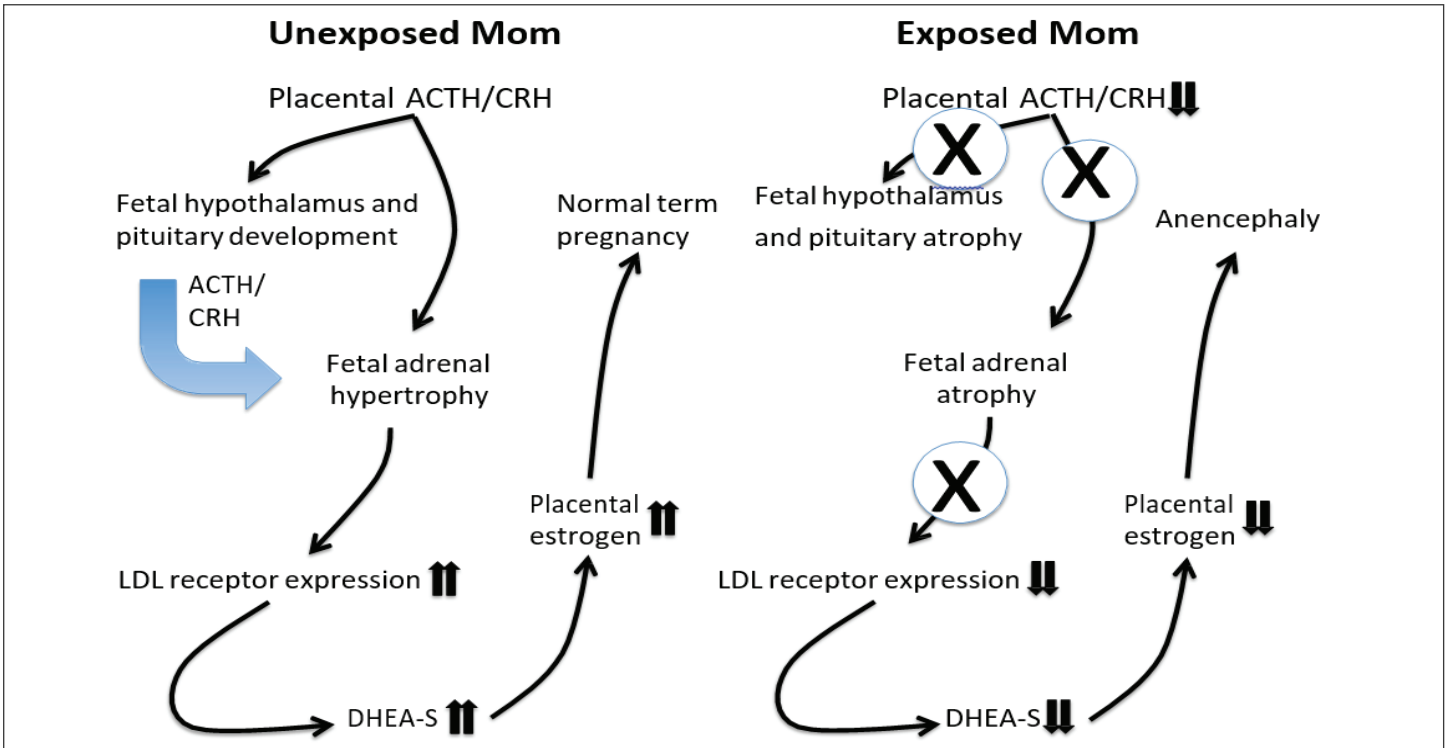

Figure 1: Schematic of hormonal disruptions in the anencephalic fetus, beginning with toxicity in the hypothalamus/pituitary glands and leading to fetal adrenal atrophy, reduced LDL receptor expression, impaired adrenal DHEA-S synthesis, and subsequent deficiency in estrogen expression in the placenta, suppressing neuronal proliferation and maturation in the brain.

supplying the terminal amide group: peptidylglycine alpha hydroxylating monooxygenase (PHM) [183], and peptidyl-alpha-hydroxyglycine alpha-amidating lyase (PAL). Copper is an essential component of both enzymes, and both enzymes also have necessary Zn(II) and Ca(II) as structural elements [184].

Glyphosate's knife cuts here in many ways. First, copper chelation, described above, reduces the activity of all copper dependent enzymes, including those needed for fetal ACTH processing. In fact, Bousquet-Moore D, et al. [184] describes the myriad pathologies associated with low dietary copper, and notes they are almost universally associated with diminished PHM and PAL activity, indicating that depletion in the diet or via chelation reduces enzyme activity. Second, as reviewed above, glyphosate chelates Zn and it also chelates calcium [185], both essential for PHM and PAL structural integrity. The final cut by glyphosate has to do with the potential for it to substitute for the terminal glycine in these propeptides, including the ACTH precursor, potentially rendering the enzymatic activity ineffectual.

Over the course of normal fetal development, under the positive influence of glucocorticoids [186], placental CRH continues to induce fetal pituitary ACTH production [187]. Even more pronounced is the placental CRH's role in stimulating fetal DHEA-S production [173]. This fetal DHEA-S undergoes aromatization to produce estradiol, production of which increases up to 1000 times in the last weeks of gestation [188]. Estradiol plays a vital role in the maintenance of a term pregnancy [189]. The anencephalic pituitary gland is uniquely deficient in its production of ACTH, while other pituitary hormones are produced at normal levels [190]. The cause of this deficient ACTH production is likely rooted in insufficient placental CRH production, inefficient ligand binding by its fetal receptor, or both. And both are susceptible to being cut by glyphosate.

Placental CRH Production

As with each amino acid, glycine has a unique transport system at the placenta and into the amniotic sac. Across the outer microvillous membrane, glycine is actively transported *via* a Na^+-coupled pump labeled System A (SA1). From there it is transported *via* System L (LAT1/LAT2) across the basement membrane into the amniotic fluid [191]. Glyphosate, likely acting as a glycine analogue [8], is taken up by the LAT1/LAT2 transport system in respiratory and gastrointestinal epithelial cells [192], and the same uptake would be expected across the amniotic membrane.

Both LAT1 and LAT2 mRNA are highly expressed in the placenta [193-195]. A study from 2011 based in Canada found that nonpregnant, but not pregnant, women had detectable levels of glyphosate in their blood [196]. This may appear as good news, but it suggests to us that the placenta may have efficiently removed glyphosate from the blood. Proliferating cells express high levels of LAT1 because they have a high demand for amino acids [197], and this means that they will absorb a higher burden of glyphosate than non-proliferating cells.

Accumulating glyphosate would be expected to be toxic to the placental cells it contacts. Richard *et al.* found placental cells to be sensitive to the toxic effects of glyphosate and, even more so, to the full complement of glyphosate plus adjuvants found in all glyphosate based herbicides [198]. This dose dependent toxicity happened at concentrations below those found in the blood of individuals occupationally exposed. Cell atrophy and death would be expected to result in both reduced CRH production and low placental weight. The former has not been directly studied vis-a-vis anencephaly, but given that placental CRH stimulates fetal ACTH production [176], and given that fetal ACTH serum levels are significantly reduced in the anencephalic fetus [199], it is plausible that glyphosate is contributing to this reduced placental CRH production. Finally, low placental weight has been documented in anencephalic births [200].

The next cut happens with the CRH receptor. In mouse and rat models, considered representative of mammalian receptor expression, CRF-1 is the dominant receptor of the fetal pituitary [201]. Substitution of glycine residues at critical positions results in a dramatic reduction in the binding affinity between CRH and the CRF-1 receptor [202,203]. Once transported *via* the LAT1 receptor into the amniotic fluid, glyphosate could then substitute for glycine during biosynthesis of the fetal CRF-1 receptor, significantly reducing the binding activity of the CRH that is already present at a reduced level.

Once placental CRH production is reduced and/or fetal CRH stimulation is impaired, a chain of events ensues that all favor the outcome of anencephaly. The presence of ACTH doubles the LDL binding capacity of the fetal adrenal tissue [204], likely because ACTH increases the number of LDL receptors on the adrenal tissue. One study found up to a 5-fold increase in LDL binding capacity in mice and rat adrenal tissue with ACTH augmentation [205]. In this regard it is interesting to note that the LDL receptor has a triple-glycine motif that is highly conserved, and that results in a >10-fold decrease in LDL binding capacity when substituted, which could happen by glyphosate [206].

Increased LDL uptake results in more cholesterol esters transported into the fetal adrenal cell interior [207]. After lysosomal degradation of the LDL particle into arachidonic acid, fatty acids and free cholesterol, a long series of enzymatic steps involving the free cholesterol within the mitochondria and Golgi apparatus leads ultimately to production of DHEA-S. Fetal DHEA-S is the fuel that feeds production of placental estrogen [175].

Given that fetal ACTH levels in anencephalic pregnancies are significantly lower than normal; and given that ACTH is required for production of LDL receptors in the fetal adrenal zone; and given that LDL cholesterol is necessary for mitochondrial production of DHEA-S; then we should expect to see both low DHEA-S and elevated LDL particles in the anencephalic fetal serum. This is precisely what is found. Fetal cord blood in anencephalic pregnancies contains LDL cholesterol levels that are up to 5 times higher than in normal pregnancies, and a significant inverse correlation was found between plasma LDL and adrenal weight [208]. Likewise, fetal plasma DHEA-S levels in normal pregnancies typically climbs to over 300 μg/mL, whereas in umbilical vein plasma DHEA-S, the compartment that should be highest as it delivers to the placenta, levels in anencephalic fetuses can be undetectable [209].

As early as 1969 it was reported that decreased placental sulfatase activity is correlated with the reduced anencephalic cord blood DHEA-S content [210]. Likewise in the fetus, sulfurylation is the primary mode of steroid hormone conjugation, as opposed to glucuronidation in the adult [211]. Laatikainen et al. found that the placenta relies more heavily on the supply of sulfated steroid from the fetus, including DHEA-S, than on placental production of those sulfated hormones [212]. Glyphosate's ability to interfere with sulfate production has been abundantly documented [71,80,213]. It is plausible that glyphosate exposure, interfering with sulfate conjugation, could help to explain the relatively normal level of fetal plasma DHEA, but the complete absence of fetal plasma DHEA-S [192]. The latter is needed almost exclusively for placental estrogen synthesis, making up at least 95% of that substrate pool [198].

Glyphosate's final cut in this regard has to do with its potential impact on fetal cortisol production. A study using an ACTH challenge in fish exposed to glyphosate and other agrochemicals found that glyphosate suppresses the cortisol response but exogenous ACTH can restore levels nearly to the level of controls. Given the lack of direct toxic effect on the adrenal cells, the authors hypothesize that glyphosate's action is at the level of the pituitary and/or hypothalamus [214]. Fetal cortisol

production is significantly lower in the anencephalic fetus [215]. Cortisol's roles in pregnancy and birth are diverse, but include organ maturation in preparation for the challenges of the postpartum world [216].

Conclusion

There are multiple pathways that lead to anencephaly, each of which is likely detrimentally affected by glyphosate. Glyphosate's known metal chelation properties and adverse effects on gut microbes leads to deficiencies in several vitamins, minerals and the amino acid methionine, all of which are linked to anencephaly. Glyphosate adversely affects serine proteases that are essential for development. Glyphosate has been shown to induce oxidative stress, and this leads to zinc sequestering by metalloproteinases and subsequent zinc deficiency. Glyphosate usage on crops correlates strongly with the worldwide diabetes epidemic, and maternal diabetes is a strong risk factor for anencephaly. Multiple hormone and enzyme dysregulations by glyphosate, along with impaired homocysteine metabolism, lead to the fetal pituitary and adrenal gland pathologies associated with anencephaly. Glyphosate's multiple impacts on proper LDL receptor integrity and overall homeostasis creates yet another risk factor. While much of the evidence remains circumstantial, we believe that a preponderance of arguments, presented here, suggests that additional research is urgently needed to confirm or deny these links.

The Precautionary Principle states that "if an action or policy has a suspected risk of causing harm to the public, or to the environment, in the absence of scientific consensus (that the action or policy is not harmful), the burden of proof that it is not harmful falls on those taking that action" [217]. Glyphosate based herbicides (GBHs) have been in use for both commercial and residential applications for nearly 45 years. The evidence reviewed in this paper offers a multitude of very specific routes by which glyphosate might be impairing embryological and fetal development in ways that significantly increase the risk of anencephaly, and epidemiological and animal studies on glyphosate support a link to impaired neurodevelopment in the brain. While an increasing number of governmental regulatory agencies around the world are imposing restrictions on the application of GBHs, the United States and other countries continue to allow widespread use of GBHs in spite of the growing safety concerns, only a small portion of which have been reviewed here. Given the gravity of anencephaly as an outcome of pregnancy, we strongly encourage regulatory agencies to follow the Precautionary Principle and suspend both commercial and residential use of GBHs until it is conclusively demonstrated that glyphosate plays no role in any of the pathways to anencephaly described in this review, nor any role in the multitude of other pathologies to which it has been compellingly linked. Until that time, we urge an informed public to keep these safety concerns in mind with respect to their own use of these chemicals and consumption of foods that may be contaminated.

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