1-4-2016

Glyphosate Induces Neurotoxicity in Zebrafish

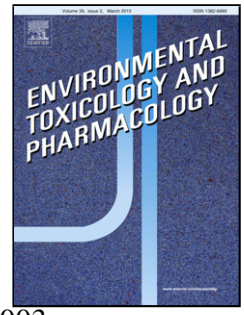
Nicole Roy
Sacred Heart University, royn@sacredheart.edu

Bruno Carneiro
Sacred Heart University, carneirob@sacredheart.edu

Jeremy Ochs
Sacred Heart University

Recommended Citation

Roy, N. M., Carneiro, B., & Ochs, J. (2016). Glyphosate Induces Neurotoxicity in Zebrafish. Environmental Toxicology and Pharmacology. doi:10.1016/j.etap.2016.01.003



Title: Glyphosate Induces Neurotoxicity in Zebrafish

Author: Nicole M. Roy Bruno Carneiro Jeremy Ochs

PII: S1382-6689(16)30003-5
DOI: http://dx.doi.org/doi:10.1016/j.etap.2016.01.003
Reference: ENVTOX 2428

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 24-10-2015
Revised date: 29-12-2015
Accepted date: 1-1-2016

Please cite this article as: Roy, N.M., Carneiro, B., Ochs, J.,Glyphosate Induces Neurotoxicity in Zebrafish, *Environmental Toxicology and Pharmacology* (2016), http://dx.doi.org/10.1016/j.etap.2016.01.003

Highlights

- Glyphosate induces loss of delineated brain ventricles and cephalic regions in zebrafish embryos
- Glyphosate decreases gene expression in the eye, fore and midbrain regions
- Glyphosate does not induce changes in the hindbrain.

Glyphosate Induces Neurotoxicity in Zebrafish

Nicole M. Roy[a], Bruno Carneiro[a], and Jeremy Ochs[a]

[a] Department of Biology, Sacred Heart University, Fairfield, CT

Corresponding Author

Nicole M. Roy

Phone: 203-365-4772

Fax: 203-365-4785

E-mail: royn@sacredheart.edu

Mailing Address: Sacred Heart University, Biology Department

5151 Park Ave, Fairfield, CT 06825

Running Head: Developmental Glyphosate Neurotoxity in Zebrafish

Keywords: Zebrafish; Development; Glyphosate; Neural; Roundup®

Abstract

Glyphosate based herbicides (GBH) like Roundup® are used extensively in agriculture as well as in urban and rural settings as a broad spectrum herbicide. Its mechanism of action was thought to be specific only to plants and thus considered safe and non-toxic. However, mounting evidence suggests that GBHs may not be as safe as once thought as initial studies in frogs suggest that GBHs may be teratogenic. Here we utilize the zebrafish vertebrate model system to study early effects of glyphosate exposure using technical grade glyphosate and the Roundup® Classic formulation. We find morphological abnormalities including cephalic and eye reductions and a loss of delineated brain ventricles. Concomitant with structural changes in the developing brain, using *in situ* hybridization analysis, we detect decreases in genes expressed in the eye, fore and midbrain regions of the brain including *pax2*, *pax6*, *otx2* and *ephA4*. However, we do not detect changes in hindbrain expression domains of *ephA4* nor exclusive hindbrain markers *krox-20* and *hoxb1a.* Additionally, using a Retinoic Acid (RA) mediated reporter transgenic, we detect no alterations in the RA expression domains in the hindbrain and spinal cord, but do detect a loss of expression in the retina. We conclude that glyphosate and the Roundup® formulation is developmentally toxic to the forebrain and midbrain but does not affect the hindbrain after 24 hour exposure.

1. Introduction

Glyphosate based herbicides (GBHs) are utilized globally and are used both in agricultural and non-agricultural (domestic and urban) areas for weed control and acts as a broad-spectrum, post-emergent herbicide (EPA; Uren Webster et al., 2014; WHO). Glyphosate is the main ingredient in formulations including Roundup®, Rodeo® and Touchdown®, each varying slightly in chemical composition and surfactant composition (Howe et al., 2004). Glyphosate strongly absorbs to soil, but it is susceptible to microbial degradation (Uren Webster et al., 2014). Due to glyphosate's low persistence, repeated applications become necessary for weed control (Ayoola, 2008). Glyphosate is also water soluble and contamination is noted during heavy rainfall. Increased river sediment loads are also noted during turbulent flooding events (Botta et al., 2009; Giesy et al., 2000; Uren Webster et al., 2014). High levels of glyphosate have also been noted in rivers near urban runoff and wastewater treatment effluent (Botta et al., 2009; Uren Webster et al., 2014). In faster moving, more diluting bodies of water, glyphosate concentrations are generally lower averaging around 10-15µg/L (Byer et al., 2008; Struger et al., 2008; Uren Webster et al., 2014). However, in stagnant bodies of water, like isolated ponds or wetlands, higher levels of glyphosate have been noted. The lack of water flow leads to less dilution and dispersion of the glyphosate (Giesy et al., 2000). Given glyphosate's high water solubility and its extensive use in the environment, exposure to non-target organisms is inevitable (Tsui and Chu, 2003). Interestingly, glyphosate nor its various formulations (with surfactants) are tested or regularly monitored in surface waters (Uren Webster et al., 2014). Glyphosate's specific mechanism of action is inhibition of the enzyme 5-*enol*pyruvoylshikimate-3-phosphate synthase (EPSPS). This plant enzyme is required in the shikimate pathway, part of the biosynthetic steps leading to formation of aromatic amino acids, but is not required in

vertebrates (Schonbrunn et al., 2001; Steinrucken and Amrhein, 1980). Thus, glyphosate was not thought to have a common molecular target in animal species (Sandrini et al., 2013). However, mounting evidence suggests non-target species may also be affected (Giesy et al., 2000).

Acute toxicity and teratogenicity in response to glyphosate was first noted in amphibian species as the nature of their reproduction and early developmental stages depends on aquatic areas making them particularly susceptible to glyphosate (Howe et al., 2004; Mann and Bidwell, 1999; Perkins et al., 2000). More recent studies focused on exposures during sensitive stages of amphibian development. Howe et al. have shown glyphosate exposure led to smaller animals than the controls as determined by decreased lengths from the snout to the vent (Howe et al., 2004). Additionally they noted delayed metamorphosis compared to controls, as well as defects in the tail regions including necrosis and blistering and abnormal gonads including intersex gonads (Howe et al., 2004). A more comprehensive study by Paganelli et al. detailed that glyphosate based herbicides induced alterations in *Xenopus* body, brain and eye development (Paganelli et al., 2010). Specifically, the authors noted alterations in neural crest development, primary neuron differentiation and loss of hindbrain rhombomere patterning using *in situ* hybridization approaches. Additionally, craniofacial and cephalic defects including reduction of the optic vesicles and microcephaly were noted that were attributable to glyphosate induced misregulation of the Retinoic Acid pathway (Paganelli et al., 2010).

There is also growing evidence that glyphosate based herbicide (GBH) toxicity is not limited to aquatic life. In rural areas, particularly in farm heavy regions of South America and Paraguay where GBHs are extensively used, an alarming trend of birth defects is starting to appear including microcrocephaly, anencephaly, cleft palates and a variety of other facial defects

(Benitez Leite et al., 2009; Campana et al., 2010). Additionally, glyphosate is used in Colombia to eradicate coca plantations. Epidemiological studies between 2004-2008 found increased rates of cyclopia at endemic levels (Lopez et al., 2012; Saldarriaga, 2010). Glyphosate has been shown to permeate the human placenta (Poulsen et al., 2009) and thus the risk of glyphosate induced teratogenesis in human development is evident.

In situ hybridization using neural specific markers is a key tool in investigating gene expression changes in response to chemical challenge. The unique patterns by which each gene is expressed allows one to investigate changes in specific areas or in multiple areas of the developing brain. For example, *krox-20* is a zinc-finger transcription factor expressed uniquely in rhombomere stripes 3 and 5 and is directly activated by *hox* genes (Giudicelli et al., 2001). *hoxb1a* is a regulatory transcription factor expressed as a single stripe in rhombomere 4 (Rohrschneider et al., 2007). Thus, the unique pattern of these genes provide information on proper hindbrain patterning. Alterations in the stripes would indicate defects in hindbrain development. *ephA4* can provide information on the developing hindbrain, forebrain and midbrain as it is expressed in multiple regions (Jessell and Sanes, 2000). Thus, *ephA4* is a good marker to investigate changes in multiple areas of the developing brain. *otx2* is a key regulator specifically in developing forebrain structures (Mori et al., 1994; Pannese et al., 1995). *pax* genes are essential transcription factors in development. Specifically, *pax6* is necessary for mammalian eye and nervous system development and acts as a master control gene which controls the development of a single eye field in the anterior neural plate into two eye fields which form the left and right optic vesicles and optic cups (Graw, 2010). Any chemical induced alterations to *pax6* expression could lead to detrimental defects in the anterior cephalic regions

and the eyes. Mutations in *pax6* are known to induce eye disorders (Bhatia et al., 2013). Likewise *pax2* plays an important role in eye development (Pfeffer et al., 1998).

Zebrafish is commonly used as a vertebrate model in developmental neurotoxicity studies given their genetic and embryological similarities to higher order vertebrate species (Dai et al., 2014; de Esch et al., 2012; Grunwald and Eisen, 2002; Hill et al., 2005; Parng et al., 2007; Teraoka et al., 2003). Zebrafish embryos are especially suited for neurotoxicological studies as fluorescent neural transgenic yield real-time phenotypes, neurons and axons are easily visualized and behavioral protocols have become well established (Linney et al., 2004; Ton et al., 2006). The zebrafish model has been used extensively to model environmental toxins including heavy metals, persistent organic pollutants and endocrine disrupting chemicals (Dai et al., 2014).

Currently, there is limited data regarding exposure to glyphosate during the windows of embryonic development. Most data in the literature is general and involves death as an end-point. Here we seek to investigate the effects of glyphosate-based herbicide exposure using technical grade glyphosate and the Roundup® Classic formulation on the developing brain using the zebrafish vertebrate toxicity model system. We investigate structural changes to the fore, mid and hindbrain by examining gross structural morphology and further investigate morphological abnormalities by investigating gene expression changes via *in situ* hybridization, immunohistological and transgenic approaches. We conclude that glyphosate and the GHB herbicide Roundup® Classic are neurotoxic to the fore and midbrain, but does not induce hindbrain changes as seen in other species.

2. Methods

2.1 Adult and Embryo handling

Wild-type AB strain and transgenic adult zebrafish were housed in a ZMOD (zebrafish module) System (Aquatic Habitats Inc.) on a 14:10 hour light:dark cycle. Adults were fed once daily with a combination of brine shrimp and supplemental TetraMin® flake food. A 10% water change was performed and water quality was monitored daily. Ammonia levels were kept below 0.5ppm, nitrate levels below 80ppm, nitrite levels below 1ppm and pH was kept between 6.5 and 7.5 values. Transgenic fish *RGYn* (Retinoic Acid Responsive Element- yellow fluorescent protein) were attained from the Linney Lab (Duke University Medical Center) (Perz-Edwards et al., 2001). Embryos were generated by natural pair-wise mating in zebrafish mating boxes (Westerfield, 1993). Embryos were placed in Petri dishes in 30% Danieau Buffer (50X Danieau's Solution [169.475g NaCl, 2.61g KCl, 4.93g $MgSO_4$ 7H20, 7.085g $Ca(NO_3)2$ $4H_20$, 0.5M Hepes at a pH of 7.6, autoclaved]. A solution of 30% Danieau's buffer was prepared by mixing 6ml of the 50X concentrated solution into 1L of distilled H_2O at 28° C for 5 hours (h) before moving into treatment. Zebrafish were staged in accordance with standard staging series (Kimmel et al., 1995). All treatments were approved and met ethical standards by the Sacred Heart University Institutional Animal Care and Use Committee (IACUC).

2.2 Solutions and exposure protocols

Embryos were collected after pair-wise male/female mating and transferred to control (30% Danieau Buffer) or 50µg/ml glyphosate concentration by diluting Roundup® (commercially purchased) or pure glyphosate (Sigma-Aldrich) in 30% Danieau Buffer at 5 hours post fertilization (hpf) (just before gastrulation) and treated continuously until 24h in development when the major brain ventricles and structures have formed and are clearly delineated visually (Figure 1). We chose the 5h-24h time window to initiate treatments at the onset of gastrulation and cover major neural developmental stages including segmentation,

somitogenesis and neurulation. Embryos were raised at 28.5ºC in standard glass petri dishes. For each type of experiment (live gross morphology, *in situ* hybridization (per gene), immunohistochemistry, transgenics) embryos were placed in the control, Roundup® dilution or pure glyphosate dilution and treated until the 24 hour time point (Figure 1). To ensure the data was not skewed by slowly developing embryos, embryos were examined for 24h hallmarks including presence of the otic vesicle, development of the lens and retina and pericardial cavity.

2.3 Live Gross Morphology

A total of ten embryos for control, Roundup® treated and glyphosate treated were tested. Thus, one experimental replicate had an n of 10. Each experiment was repeated three separate times on different days on different embryo clutches so the total tested for control and treated was 30 (n= 10 per experiment, replicate =3, total n=30). Live images were taken under a Leica dissection microscope attached to a digital camera using QCapture Software. Embryos were placed in 3% methylcellulose for positioning purposes in a depression slide. Tricaine methanesulfonate (MS-222) (Westerfield, 1993) was used to anesthetize highly mobile embryos.

2.4 Transgenics

Retinoic Acid response elements were engineered with a basal promoter and fused with enhanced yellow fluorescence protein to yield the transgenic *RGYn* fish utilized in the experiments (Perz-Edwards et al., 2001). Transgenic embryos are particularly useful to study development, as results are seen in real time, *in vivo* by simply viewing the live control and treated embryos under fluorescent microscopy. Thus, there is no need for fixation required for in situ processing or immunohistochemistries. Fluorescent images were taken using the above embryo protocols using a Nikon Eclipse E400 fluorescent microscope. Transgenics were treated

with phenylthiourea to prevent melanin formation which would obscure fluorescent signal (Westerfield, 1993). A total of ten embryos for control, Roundup® treated and glyphosate treated were analyzed for all live and transgenic embryos. Each experiment was repeated three times. The total n for each (control and treated) was 30.

2.5 in situ hybridization (per gene)

Protocols for *in situ* hybridization were following according to Sagerstrom et. al (Sagerstrom et al., 1996). All probes used (*pax2, pax6, otx2, ephA4, hoxb1a* and *krox-20*) were generous gifts from the Sagerstrom Lab (UMASS Medical Center, Worcester, MA). All probes were Digoxigenin (DIG) labeled (Roche Life-Sciences) antisense RNA probes transcribed using the SP6/T7 *in vitro* transcription kit (Promega). The hybridized probes were visualized in blue color using an anti-DIG antibody (Roche Life Sciences) bound to nitroblue tetrazolium and bromo-4-chloro-indolyl phosphate (NBT/BCIP, Promega). Controls and treated embryos were run side-by-side in separate wells in the same solutions, thus all solutions were normalized between the samples. A total of ten 24h embryos for control, Roundup® treated and glyphosate treated were analyzed for changes in *in situ* staining/expression. The experiment was repeated three times. The total n for each (control and treated) was 30.

2.6 Whole mount immunohistochemistry

Whole mount immunohistochemistries were performed as previously described (Barresi et al., 2001; Devoto et al., 1996). A zn-8 antibody was obtained from Developmental Studies Hybridoma Bank at the University of Iowa. A 1:5 dilution of supernatant zn-8 antibody was utilized. FITC-labeled goat anti-mouse secondary antibody (Santa Cruz Biotechnology) was utilized at a 1:200 dilution. Controls and treated embryos were run side-by-side in separate wells

in the same solutions and time frames. Embryos were imaged as described above using the FITC fluorescent filter cube. The experiment was repeated three times. The total n for each (control and treated) was 30.

3. Results

3.1 General Live Brain Morphology

To investigate general *in vivo* neural structural changes, embryos were examined at 24h of development when the major brain ventricles have developed and have become clearly delineated visually. Phenotypes for the 50µg/ml glyphosate concentration by diluting the Roundup® and the pure glyphosate treatment yielded a range of phenotypes from severe to mild. In lateral views, control embryos demonstrate very clear delineations between the forebrain (FB), midbrain (MB), mid-hindbrain boundary (MHB), hindbrain (HB) and otic vesicle (OV) positioned just outside rhombomere (r) 4 (Figure 2A). In frontal views, the MHB and forebrain ventricle, both the diencephalic (di) and telencephalic (tel) portions are clearly seen along with the lens and retina of the eyes (Figure 2E). In only 10% (3/30) of the Roundup® treated and 13.3% (4/30) of the glyphosate treated embryos were all brain ventricles and structures clearly visible (Figure 2B,F). In 60% (18/30) of the Roundup® treated and in 66.6% (20/30) of the glyphosate treated embryos, a loss of identifiable ventricles was seen, where only the MHB and otic vesicle could be visualized in lateral views (Figure 2C). A complete loss of delineated brain ventricles, with no identifiable structures other than the otic vesicle outside r4 was seen in 30% (9/30) of the Roundup® treated and 20% (6/30) of the glyphosate treated embryos in lateral

views (Figure 2D). In frontal views of these embryos, the brain appears flattened and ventricles unidentifiable (Figure 2H). The eyes also appear smaller.

3.2 in situ hybridization

Since we detected a loss of brain ventricle delineations and a general cephalic reduction was noted, *in situ* hybridizations were performed using genes that are expressed in the forebrain, midbrain hindbrain and eye, either in those specific ventricles alone or in multiple ventricle regions.

pax 2

pax2 (paired box gene 2) is an essential transcription factor expressed strongly in the anterior retina (AR) (Figure 3A,B,C), the MHB (Figure 3A,B,D,E), the optic stalk (OS) (Figure 3C), the otic vesicle (OV) (Figure 3B,D) and weakly expressed throughout the hindbrain (HB) and spinal cord (SC) in control embryos (Figure 3B,D) (Pfeffer et al., 1998). Treated embryos demonstrate a decrease or alteration of *pax2* expression. Treated embryos show a decreased and flattened anterior retina (Roundup®: 90% (27/30), glyphosate: 26/30 (86.6%)) with concomitant loss of the choroid fissure (Figure 3H). The mid-hindbrain boundary demonstrates a loss of the high apex cone shape, demonstrating a flattened, more rounded MHB (Figure 3J) (Roundup®: 93.3% (28/30), glyphosate: 100% (30/30)). However, the *pax2* staining on the otic vesicle was unchanged (Roundup®: 96.7% (29/30), glyphosate: 96.7% (29/30)) expressing *pax2* normally. Additionally, the weaker hindbrain and spinal cord *pax2* expression appeared normal as in control.

pax 6

pax 6 (paired box gene 6) is an essential transcription factor for eye development (Nornes et al., 1998). In control embryos, *pax 6* is expressed strongly in the forebrain region, specifically the dorsal diencephalon (ddi) (Figure 4A-E), in both the lens and retina of the eye (Figure 4A-E) and broadly in the hindbrain and spinal cord (Figure 4A,B,D). Notably, there is no expression in the telencephalic portion of the forebrain (Figure 4C). Thus a dome of *pax 6* expression can be seen in the frontal view (Figure 4C). In treated embryos, a decrease in *pax 6* expression in seen in the dorsal diencephalon of treated embryos (Roundup®: 90% (27/30), glyphosate: 96.6% (29/30)) and a decrease in the eye is seen (Roundup®: 93.3% (28/30), glyphosate: 96.6% (29/30)) (Figure 4J). Additionally, the domed *pax 6* expression seen in the controls in frontal view (Figure 4C) is not seen in treated embryos, where it appears the brain has flattened and lost the more anterior telencephalic portion of the forebrain (Figure 4H). In contrast, a decrease in the hindbrain and spinal cord staining is only seen in 6.6% (2/30) of the Roundup® treated and 13/3% (4/30) of the glyphosate treated embryos and appears normal.

otx2

otx2 is expressed in the brain, specifically the diencephalon and mesencephalon as well as the eye (Mori et al., 1994; Pannese et al., 1995) and is a key regulator in patterning of anterior neural structures (Pannese et al., 1995; Scholpp et al., 2007). In control embryos, *otx2* is expressed in the dorsal diencephalic (ddi) portion of the forebrain and the ventral midbrain (vm) (Figure 5A-E). In treated embryos a decrease in *otx2* expression is seen in the dorsal diencephalon and midbrain regions (Roundup®: 93.3% (28/30), glyphosate: 90% (27/30)) (Figure 5F-J). In close-up views, *otx2* demonstrates a butterfly pattern in the dorsal diencephalon and midbrain (Figure 5E), but that pattern is lost in treated embryos (Figure 5J).

ephA4

Segmentation of the hindbrain is established through the interaction of Eph receptors and Ephrin ligands (Jessell and Sanes, 2000). In control embryos *ephA4* (Ephrin type A Receptor) is expressed in the forebrain ventricular zone (vtz) of the diencephalon (di), the anterior midbrain and hindbrain rhombomeres r3 and r5 (Figure 6A-E). In treated embryos, a decrease in *ephA4* staining is seen in the forebrain ventricular zone (Roundup®: 90% (27/30), glyphosate: 96.6% (29/30)) and in the anterior midbrain region (Roundup®: 86.6% (26/30), glyphosate: 93.3% (28/30)) (Figure 6F-J). Interestingly, only 3.3% (1/30) of the Roundup® treated embryos and 0% (0/30) of the glyphosate treated embryos demonstrate a loss of *ephA4* staining in hindbrain rhombomeres r3 or r5 (Figure 6F,G,I) and thus in contrast to the forebrain and midbrain, the hindbrain staining appears normal.

hoxb1a and krox-20

hoxb1a and *krox-20* are exclusively hindbrain markers. The earliest rhombomere to develop and differentiate, r4, is controlled primarily by the expression of *hoxb1a* (Rohrschneider et al., 2007) and is highly Retinoic Acid sensitive. *hoxb1a* is expressed as a single stripe in rhombomere 4 and no change is seen in control (Figure 7A-D) or in Roundup® treated embryos. In 1/30 (3%) of the glyphosate treated embryos was the stripe partially missing on the left side (Figure 7E-H). *krox-20* is a zinc-finger transcription factor expressed in both rhombomere 3 and 5 and is directly activated by *hox* genes (Giudicelli et al., 2001; Wassef et al., 2008). No change is seen in control (Figure 7I-L) or in either Roundup® or glyphosate treated embryos (Figure 7M-P).

3.3 Retinoic acid response element transgenics

In frog species it is noted that Roundup® treatments induced shortening of the anterior-posterior axis and loss of anterior hindbrain rhombomeres due to increased retinoic acid (RA) activity

(Carrasco, 2013; Paganelli et al., 2010). We saw similar forebrain and midbrain effects as well as microcephaly (Figure 2-6) as noted in frogs, but did not note any hindbrain changes in our *in situ* hybridizations (Figure 6 and 7). Thus, we utilized a Retinoic Acid response element transgenic (RGYn2) to determine if in zebrafish alterations in the RA domains were also apparent. We find that there is no change or increase in the RA responsive domains in the hindbrain and spinal cord in control or treated embryos (Figure 8A,C). The signal is also apparent in the eye, strongly in the ventral retina, but weakly in the dorsal retina in control embryos (Figure 8B). In treated embryos, interestingly, there is no signal in either the dorsal or ventral retina (Roundup®: 86.6% (26/30), glyphosate: 90% (27/30)) (Figure 8D).

3.4 zn-8 Whole Mount Immunohistochemistry

As we detected microphthalmia in our general investigation of *in vivo* brain morphology (Figure 2), alterations in *pax6* expression in the eye in our *in situ* hybridizations (Figure 4) and a loss of the RGYn2 transgenic signal in the dorsal and ventral retina (Figure 8) in treated embryos, we sought to investigate if the optic nerve was also affected. We performed a whole mount immunohistochemistry using a zn-8 antibody which labels the optic nerve, optic chiasm and the bifurcation of the optic nerve into the retinal ganglion cells. These are clearly seen in control embryos (Figure 9A), but a severe loss of signal in the optic nerve and chiasm was detected in treated embryos (Roundup®: 80% (24/30), glyphosate: 73.3% (22/30)) (Figure 9B). In some cases, no signal at all was detected (Roundup®: 20% (6/30), glyphosate: 26.6% 8/30)).

4. Discussion

Concentrations of glyphosate in the literature vary. Sperm quality was assessed in fish after 96h exposure to 5-10mg/L glyphosate (Lopes et al., 2014). In another study, zebrafish were exposed for 21 days with up to 10mg/L glyphosate to investigate egg production (Uren Webster et al., 2014). One study did test newly fertilized zebrafish embryos (1.5h) using 0-150µg/ml glyphosate and studied general body toxicity up to 96h (Bortagaray et al., 2010). We performed a similar study using Roundup® Classic and technical grade glyphosate utilizing concentrations of glyphosate between 0-150µg/ml diluted in 30% Danieau Buffer and found 100% lethality at 75µg/ml and above. At 50µg/ml, embryos demonstrated no generalized necrosis, no developmental delays and no gross malformations, but a neural structural phenotype was evident. Concentrations between 50 and 75µg/ml demonstrated developmental delays and general necrosis as seen visually and as indicated using Acridine Orange staining. Furthermore, embryos treated with 50µg/ml reached 24h developmental hallmarks in sync with controls. At lower concentrations, no phenotype was visually seen. We sought to springboard off the work of Paganelli et al. who investigated a sub-lethal concentration of glyphosate and noted alterations in the brains of frogs (Paganelli et al., 2010). Although their concentration was lethal in zebrafish embryos, a lower concentration of 50µg/ml demonstrated a neural phenotype. A comprehensive study of environmental concentrations has been reported for a variety of water settings including ponds, seawater, surface water, mudflats, creeks and streams (Giesy et al., 2000).

There is very little in the literature regarding glyphosate and zebrafish. A study has been performed to assess the effect of glyphosate exposure on reproduction in adults. In the study, breeding adults were exposed to glyphosate for 21 days and it was determined that glyphosate reduced egg production, but had no effect on fertilization rates in breeding colonies. Early staged embryo mortality and premature hatching was noted and attributed to exposure during

gametogenesis (Uren Webster et al., 2014). The effect of glyphosate on sperm production and egg quality has also been studied. It was determined that glyphosate induced decreases in sperm motility and mitochondrial functionality. Additionally, defects in membrane and DNA integrity were noted (Lopes et al., 2014). Glyphosate has also been determined to increase the diameter of oocytes (Armiliato et al., 2014). To date, there has been no study on glyphosate and its effect on the development of the zebrafish embryo.

Additionally, there have been relatively few in-depth publications on the embryonic toxicity of glyphosate, mostly studies note general death, but not specific morphological alterations or gene and protein changes. The most comprehensive studies have been performed in frog species (Howe et al., 2004; Paganelli et al., 2010). Some early studies demonstrated that chronic exposure to tadpoles with Roundup® Classic showed decreased snout-vent length, delayed developmental hallmarks, necrotic and blistered tails and gonadal abnormalities in four North American frog species (Howe et al., 2004). Another study detailed the LC_{50}s of Roundup® Classic on 13 species of larval amphibians (Relyea and Jones, 2009). Recently, a more investigative study was published that examined the effects of a 1:5000 dilution of Roundup® Classic (glyphosate at 72 µg/ml) on *Xenopus laevis* embryonic development with some additional data presented on chick. It was found that exposure shortened the anterior-posterior (A-P) axis (Paganelli et al., 2010). As the focus of our study was strictly the brain, we did not measure the total embryo length, choosing to study the brain regions using specific markers, however, we did not note any observable changes in the length of zebrafish embryos developing at 50µg/ml (Figures 3-8, full body images). Furthermore, Paganelli investigated hindbrain marker *krox-20,* which is expressed in rhombomeres 3 and 5 in frog and noted a loss of the r3 stripe. The authors correlate the loss of r3 *krox-20* staining to loss of anterior

rhombomeres occurring due to increased concentrations of RA (Paganelli et al., 2010). Additionally, Paganelli et. al. noted in chick a decrease in expression of Pax6 antibody expression in r3 and r5 (in chick, *pax6* is expressed in rhombomere 3 and 5, but not in frog or zebrafish). We tested three genes expressed in the hindbrain, *ephA4, krox-20* and *hoxb1a*. Interestingly, we find no effect on the expression of these genes in treated embryos demonstrating no decrease along the A-P axis of the hindbrain. Additionally, no change in the location of the OV found just lateral to r4 was noted. In the same frog study, Paganelli et. al. noted cephalic reductions and microphthalmy and thus investigated genes downstream of the sonic hedgehog signaling pathway, *pax6* and *otx2*. They noted a distinct down-regulation of *pax6* expression in the eye region and a reduction of *otx2* in its expression domain. Here we find similar results morphologically in terms of cephalic reductions and microphthalamy (Figure 2-9).

Furthermore, Paganelli et. al. determined that the phenotypes seen in their frog experiments (shortened A-P axis, loss of anterior rhombomeres, cephalic reductions, microphthalmy) were mediated by altered Retinoic Acid signaling. Upon closer examination, they determined through a reporter assay that the levels of RA in treated embryos were significantly increased (Paganelli et al., 2010). Interestingly, in contrast to their study, we do not find a loss of hindbrain specific genes sensitive to RA (*hoxb1a, krox-20, ephA4).* We further utilized a Retinoic Acid mediated transgenic reporter fish (Perz-Edwards et al., 2001) to determine if exposure to Roundup® or glyphosate caused an upregulation of RA signaling. We found no change in the RA responsive domain in the hindbrain and spinal cord, but do find a difference in the eye (Figure 8). This agrees with our results in that no hindbrain markers which are sensitive to RA were affected (*ephA4, krox-20, hoxb1a*), but several genes expressed in the

eye did show down-regulation (*pax6, pax2*) and a loss of the zn-8 antibody staining was also seen.

Of particular note, the changes in gene expression we detected are not attributable to delayed development. Treated embryos met developmental milestones accordingly and in sync with control treatments at 50µg/ml. Thus, it does not appear that in zebrafish, at the concentration tested, that Roundup® Classic or glyphosate causes defects in the hindbrain, but may be reason for the anterior neural and eye changes we detect. Time-wise, treatments in *Xenopus* were similar to our zebrafish study with gene expression analyzed at the neurula and tailbud stages (Paganelli et al., 2010). To determine the effect of Roundup® or glyphosate on early neural development in zebrafish, we chose a treatment window that included segmentation, somitogenesis and neurulation (5h-24h).The zebrafish brain is patterned and established by 24h and hence why this was chosen as an endpoint (Appel, 2000; Kimmel, 1993). In future studies, later staged defects may be seen, but that was beyond the scope of this study.

Glyphosate based herbicides like Roundup® Classic often contain a mixture of chemicals, mainly glyphosate mixed with surfactants to aid in leaf retention and absorption. A common additive is polyethoxylated tallow amine (POEA) (Brausch and Smith, 2007; Giesy et al., 2000) and is present in the Roundup® Classic formulation used in this study. Thus, glyphosate formulations like Roundup®, Rodeo®, Touchdown® could be more toxic than pure glyphosate by the addition of surfactants (Howe et al., 2004; Mann and Bidwell, 1999). However, recent experiments in frog using treatments with Roundup® classic as a GBH with POEA or pure glyphosate without added surfactants yielded similar phenotypes (Paganelli et al., 2010). Additionally, our results using Roundup® or pure technical grade glyphosate yielded the

same results eliminating the possibility that Roundup® is more toxic due to the addition on the surfactant.

As the only other zebrafish papers investigating the effect of glyphosate based herbicides are in adults, this appears to be one of the first studies noting the developmental neurotoxicity of glyphosate in zebrafish. Here we find that similar to frog species, Roundup® Classic or glyphosate induces cephalic reductions and microphthalamy, but in contrast to what is found in frog, does not appear to affect the hindbrain. In the frog experiment, the cephalic malformations and body shortening with concomitant loss of gene expressions (*otx2, pax6, krox-20*) can be explained by an excess is RA signaling (Paganelli et al., 2010). Although we detect similar changes is the forebrain, midbrain and eye neural structures, we do not detect a change in the hindbrain. This brings up potential interesting differences in the mechanism by which glyphosate increases endogenous RA activity. We clearly see changes in RA sensitive areas in the eye with the RGYn transgenic, but no change in the RA sensitive regions in the hindbrain and spinal cord was visually observed (Figure 8). However, the RA synthesizing activity in the retina and hindbrain/spinal cord could be under the control of different RA synthases and RA degrading enzymes underlying the difference we see in response to glyphosate in the different species. However, as Retinoic Acid signaling relies on a complex interplay between receptors, coactivators and antagonizing proteins (Perz-Edwards et al., 2001), a complete analysis of this was beyond the scope of this study. Thus, although this study provides preliminary information on the developmental neurotoxicity of glyphosate, much remains to be elucidated mechanistically as to why the toxic effect appears to be specific to the fore and midbrain, but not the hindbrain in zebrafish.

5. Conclusion

Glyphosate based herbicides are extensively used globally and there is little in the literature investigated neurotoxicity of this chemical to vertebrate species. Here, we utilize the zebrafish model system to investigate neurotoxicity and find morphological changes in brain architecture including loss of delineated brain ventricles and reductions in cephalic and eye regions. Utilizing in situ hybridization techniques with cephalic and eye markers including *pax2, pax6, otx2, ephA4* and immunohistochemistries with an optic nerve antibody zn-8 to more specifically pin-point changes, we find decreases in expression of cephalic and eye markers, but do not detect changes in the hindbrain region as detected by *ephA4, hoxb1a* and *krox-20* in situ staining. This suggests that in zebrafish, glyphosate in neurotoxic to the forebrain and midbrain regions by altering expression of key gene regulators in development, but does not affect the hindbrain.

Acknowledgements

The authors would like to thank the Sagerstrom Lab (UMASS Medical School) for generous donation of plasmids used to generate *in situ* probes and the Linney Lab (Duke University Medical Center) for generous donation of the RGYn2 transgenic zebrafish.

References

Appel, B. (2000). Zebrafish neural induction and patterning. *Dev Dyn* **219**(2), 155-68.

Armiliato, N., Ammar, D., Nezzi, L., Straliotto, M., Muller, Y. M., and Nazari, E. M. (2014). Changes in ultrastructure and expression of steroidogenic factor-1 in ovaries of zebrafish Danio rerio exposed to glyphosate. *J Toxicol Environ Health A* **77**(7), 405-14.

Ayoola, S. O. (2008). Toxicity of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*) juvenile. *African Journal of Agricultural Researhc* **3**(12), 825-834.

Barresi, M. J., D'Angelo, J. A., Hernandez, L. P., and Devoto, S. H. (2001). Distinct mechanisms regulate slow-muscle development. *Curr Biol* **11**(18), 1432-8.

Benitez Leite, S., Macchi, M. A., and Acosta, M. (2009). Malformaciones Congenitas asociadas a agrotoxicos. *Arch. Pediatr Drug* **80**, 237-247.

Bhatia, S., Bengani, H., Fish, M., Brown, A., Divizia, M. T., de Marco, R., Damante, G., Grainger, R., van Heyningen, V., and Kleinjan, D. A. (2013). Disruption of autoregulatory feedback by a mutation in a remote, ultraconserved PAX6 enhancer causes aniridia. *Am J Hum Genet* **93**(6), 1126-34.

Bortagaray, V., Cruces Aramburu, R., Barrios, L., Ojeda, P., del Puerto, G., and Rodriguez-Ithurralde, D. (2010). Embryotoxicity and teratogenesis in zebrafish embryos exposed in vitro to glyphosate-type herbicides. *Journal of Developmental Toxicity*.

Botta, F., Lavison, G., Couturier, G., Alliot, F., Moreau-Guigon, E., Fauchon, N., Guery, B., Chevreuil, M., and Blanchoud, H. (2009). Transfer of glyphosate and its degradate AMPA to surface waters through urban sewerage systems. *Chemosphere* **77**(1), 133-9.

Brausch, J. M., and Smith, P. N. (2007). Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, Thamnocephalus platyurus. *Arch Environ Contam Toxicol* **52**(2), 217-21.

Byer, J. D., Struger, J., Klawunn, P., Todd, A., and Sverko, E. (2008). Low cost monitoring of glyphosate in surface waters using the ELISA method: an evaluation. *Environ Sci Technol* **42**(16), 6052-7.

Campana, H., Pawluk, M. S., and Lopez Camelo, J. S. (2010). [Births prevalence of 27 selected congenital anomalies in 7 geographic regions of Argentina]. *Arch Argent Pediatr* **108**(5), 409-17.

Carrasco, A. (2013). *Teratogenesis by glyphosate based herbicides and other pesticides. Relationship with the retinoic acid pathway*, Frankfurt.

Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., Chen, Y. L., and Pei, D. S. (2014). Zebrafish as a model system to study toxicology. *Environ Toxicol Chem* **33**(1), 11-7.

de Esch, C., Slieker, R., Wolterbeek, A., Woutersen, R., and de Groot, D. (2012). Zebrafish as potential model for developmental neurotoxicity testing: a mini review. *Neurotoxicol Teratol* **34**(6), 545-53.

Devoto, S. H., Melancon, E., Eisen, J. S., and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122**(11), 3371-80.

EPA, U. S. Available at: http://www.epa.gov/ogwdw/pdfs/factsheets/soc/tech/glyphosa.pdf.

Giesy, J. P., Dobson, S., and Solomon, K. R. (2000). Ecotoxicological Risk Assessment for Roundup Herbicide. *Rev Environ Contam Toxicol* **167**, 35-120.

Giudicelli, F., Taillebourg, E., Charnay, P., and Gilardi-Hebenstreit, P. (2001). Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev* **15**(5), 567-80.

Graw, J. (2010). Eye development. *Curr Top Dev Biol* **90**, 343-86.

Grunwald, D. J., and Eisen, J. S. (2002). Headwaters of the zebrafish -- emergence of a new model vertebrate. *Nat Rev Genet* **3**(9), 717-24.

Hill, A. J., Teraoka, H., Heideman, W., and Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci* **86**(1), 6-19.

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., and Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environ Toxicol Chem* **23**(8), 1928-38.

Jessell, T. M., and Sanes, J. R. (2000). Development. The decade of the developing brain. *Curr Opin Neurobiol* **10**(5), 599-611.

Kimmel, C. B. (1993). Patterning the brain of the zebrafish embryo. *Annu Rev Neurosci* **16**, 707-32.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* **203**(3), 253-310.
Linney, E., Upchurch, L., and Donerly, S. (2004). Zebrafish as a neurotoxicological model. *Neurotoxicol Teratol* **26**(6), 709-18.
Lopes, F. M., Varela Junior, A. S., Corcini, C. D., da Silva, A. C., Guazzelli, V. G., Tavares, G., and da Rosa, C. E. (2014). Effect of glyphosate on the sperm quality of zebrafish Danio rerio. *Aquat Toxicol* **155**, 322-6.
Lopez, S. L., Aiassa, D., Benitez Leite, S., Lajmanovich, R., Manas, F., Poletta, G., Sanchez, N., Simoniello, M. F., and Carrasco, A. E. (2012). *Pesticides Used in South American GMO-Based Agriculture: A Review of Their Effects on Humans and Animal Models*. Elsevier.
Mann, R. M., and Bidwell, J. R. (1999). The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch Environ Contam Toxicol* **36**(2), 193-9.
Mori, H., Miyazaki, Y., Morita, T., Nitta, H., and Mishina, M. (1994). Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res Mol Brain Res* **27**(2), 221-31.
Nornes, S., Clarkson, M., Mikkola, I., Pedersen, M., Bardsley, A., Martinez, J. P., Krauss, S., and Johansen, T. (1998). Zebrafish contains two pax6 genes involved in eye development. *Mech Dev* **77**(2), 185-96.
Paganelli, A., Gnazzo, V., Acosta, H., Lopez, S. L., and Carrasco, A. E. (2010). Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. *Chem Res Toxicol* **23**(10), 1586-95.
Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**(3), 707-20.
Parng, C., Roy, N. M., Ton, C., Lin, Y., and McGrath, P. (2007). Neurotoxicity assessment using zebrafish. *J Pharmacol Toxicol Methods* **55**(1), 103-12.
Perkins, P. J., Boermans, H. J., and Stephenson, G. R. (2000). Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay-Xenopus. *Environ Toxicol Chem* **19**, 940-945.
Perz-Edwards, A., Hardison, N. L., and Linney, E. (2001). Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev Biol* **229**(1), 89-101.
Pfeffer, P. L., Gerster, T., Lun, K., Brand, M., and Busslinger, M. (1998). Characterization of three novel members of the zebrafish Pax2/5/8 family: dependency of Pax5 and Pax8 expression on the Pax2.1 (noi) function. *Development* **125**(16), 3063-74.
Poulsen, M. S., Rytting, E., Mose, T., and Knudsen, L. E. (2009). Modeling placental transport: correlation of in vitro BeWo cell permeability and ex vivo human placental perfusion. *Toxicol In Vitro* **23**(7), 1380-6.
Relyea, R. A., and Jones, D. K. (2009). The toxicity of Roundup Original Max to 13 species of larval amphibians. *Environ Toxicol Chem* **28**(9), 2004-8.
Rohrschneider, M. R., Elsen, G. E., and Prince, V. E. (2007). Zebrafish Hoxb1a regulates multiple downstream genes including prickle1b. *Dev Biol* **309**(2), 358-72.
Sagerstrom, C. G., Grinbalt, Y., and Sive, H. (1996). Anteroposterior patterning in the zebrafish, Danio rerio: an explant assay reveals inductive and suppressive cell interactions. *Development* **122**(6), 1873-83.
Saldarriaga, W. (2010). Epidemiological surveillance of cyclopia in the Hospital Universitario del Valle, Cali, Colombia 2004-2008. *Revista Colombiana De Obstetricia Y Ginecologia* **61**, 12-17.
Sandrini, J. Z., Rola, R. C., Lopes, F. M., Buffon, H. F., Freitas, M. M., Martins Cde, M., and da Rosa, C. E. (2013). Effects of glyphosate on cholinesterase activity of the mussel Perna perna and the fish Danio rerio and Jenynsia multidentata: in vitro studies. *Aquat Toxicol* **130-131**, 171-3.
Scholpp, S., Foucher, I., Staudt, N., Peukert, D., Lumsden, A., and Houart, C. (2007). Otx1l, Otx2 and Irx1b establish and position the ZLI in the diencephalon. *Development* **134**(17), 3167-76.

Schonbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V., Amrhein, N., Evans, J. N., and Kabsch, W. (2001). Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc Natl Acad Sci U S A* **98**(4), 1376-80.
Steinrucken, H. C., and Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. *Biochem Biophys Res Commun* **94**(4), 1207-12.
Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., and Marvin, C. (2008). Occurrence of glyphosate in surface waters of Southern Ontario. *Bull Environ Contam Toxicol* **80**(4), 378-84.
Teraoka, H., Dong, W., and Hiraga, T. (2003). Zebrafish as a novel experimental model for developmental toxicology. *Congenit Anom (Kyoto)* **43**(2), 123-32.
Ton, C., Lin, Y., and Willett, C. (2006). Zebrafish as a model for developmental neurotoxicity testing. *Birth Defects Res A Clin Mol Teratol* **76**(7), 553-67.
Tsui, M. T., and Chu, L. M. (2003). Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere* **52**(7), 1189-97.
Uren Webster, T. M., Laing, L. V., Florance, H., and Santos, E. M. (2014). Effects of glyphosate and its formulation, roundup, on reproduction in zebrafish (Danio rerio). *Environ Sci Technol* **48**(2), 1271-9.
Wassef, M. A., Chomette, D., Pouilhe, M., Stedman, A., Havis, E., Desmarquet-Trin Dinh, C., Schneider-Maunoury, S., Gilardi-Hebenstreit, P., Charnay, P., and Ghislain, J. (2008). Rostral hindbrain patterning involves the direct activation of a Krox20 transcriptional enhancer by Hox/Pbx and Meis factors. *Development* **135**(20), 3369-78.
Westerfield, M. (1993). *The Zebrafish Book: A guide for the laboratory use of zebrafish (brachydanio rerio)*. Univ. of Oregon Press, Eugene OR.
WHO. Available at: http://www.who.int/ipcs/publications/ehc/ehc_numerical/en/.

Figure Legends

Fig. 1. Schematic overview of experimental set-up. A. Wild type male and female fish were mated. At 5 hours of development, embryos were separated and treated with the control Danieau Buffer or the glyphosate formulation. At 24 hours of development the live embryos were analyzed under dissection microscopy for alterations in neural anatomy or processed for in situ hybridization or immunohistochemistry. B. RGYn transgenic male and female fish were mated. At 5 hours of development, embryos were separated and treated with the control Danieau Buffer or the glyphosate formulation. At 24 hours of development the live embryos were analyzed under fluorescent microscopy for alterations in the Retinoic Acid responsive domains as indicated by the yellow fluorescent protein expression.

Fig. 2. Live images of control and treated embryos at 24hrs. Shown for treated embryos are the Roundup® treatments, as the Roundup® and glyphosate treatment yielded the same phenotypes. (A-D) lateral views, (E-H) frontal views. Control (A,E) embryos demonstrate normal neural architecture including clear delineations between the forebrain, midbrain and hindbrain. The otic vesicle is clearly seen outside rhombomere 4. The lens and retina are also normal (E). Treated embryos showing mild (B,F), moderate (C,G) and severe (D,H) loss of brain delineations, but a normal otic vesicle. In frontal views, the treated brains are flattened and eyes appear smaller. FB: forebrain, MB: midbrain, MHB: mid-hindbrain boundary, HB: hindbrain, OV: otic vesicle, FB(di): diencephalic portion of forebrain, FB(tel): telencephalic portion of forebrain

Fig. 3. *pax2 in situ* hybridization at 24hrs. (A,F) whole body lateral views, (B,G) lateral view of head (C,H) frontal views, (D,I) dorsal views, (E,J) magnified view of MHB. Control embryos (A-E) demonstrate strong *pax2* staining in the anterior retina, the mid-hindbrain boundary, optic stalk, and otic vesicle. *pax2* is weakly expressed throughout the hindbrain and spinal cord. Treated embryos (F-J) demonstrate a decrease and flattened anterior retina with loss of staining in the choroid fissure. The mid-hindbrain boundary has lost the normal apex, demonstrating a flattened and more rounded shape. *pax2* expression in the otic vesicle, hindbrain and spinal cord appear normal. AR: anterior retina, MHB: mid-hindbrain boundary, HB: hindbrain, SC: spinal cord, PND: pronephritic duct, OV: otic vesicle, OS: optic stalk, CF: choroid fissure

Fig. 4. *pax6 in situ* hybridization at 24hrs. (A,F) whole body lateral views, (B,G) lateral view of head (C,H) frontal views, (D,I) dorsal views, (E,J) magnified view of the eyefield and forebrain. Control embryos (A-E) demonstrate strong *pax 6* expression in the dorsal diencephalon of the forebrain, the lens and the retina and broadly in the hindbrain and spinal cord. Treated embryos (F-J) demonstrate a decrease of *pax 6* expression in the dorsal diencephalon and decreased

expression in the eye. A loss of the telencephalic portion of the brain is noted due to the loss of the dome shaped expression pattern (compare C,H). Hindbrain and spinal cord expression appear normal. FB(ddi): dorsal diencephalic portion of the forebrain, MHB: mid-hindbrain boundary, HB: hindbrain, SC: spinal cord

Fig. 5. *otx2 in situ* hybridization at 24hrs. (A,F) whole body lateral views, (B,G) lateral view of head (C,H) frontal views, (D,I) dorsal views, (E,J) magnified view of forebrain and midbrain. Control embryos (A-E) demonstrate expression in the dorsal diencephalon and the ventral midbrain. Treated embryos (F-J) demonstrate a decrease in *otx2* expression in the dorsal diencephalon and midbrain regions. FB(ddi): dorsal diencephalic portion of the forebrain, MB: midbrain, vm: ventral midbrain

Fig. 6. *ephA4 in situ* hybridization at 24hrs. (A,F) whole body lateral views, (B,G) lateral view of head (C,H) frontal views, (D,I) dorsal views, (E,J) magnified view of forebrain and midbrain. Control embryos (A-E) demonstrate *ephA4* staining in the forebrain ventricular zone of the diencephalon, the anterior midbrain and rhombomeres 3 and 5 of the hindbrain. Treated embryos (F-J) demonstrate decreased *ephA4* expression in the forebrain ventricular zone and in the anterior midbrain. However, no loss of ephA4 expression was seen in hindbrain rhombomeres 3 and 5. FB(di-vtm): ventral tegmental zone in diencephalic portion of forebrain, MB(ant): anterior portion of midbrain, r: rhombomere

Fig. 7. *hoxb1a and krox-20 in situ* hybridization at 24hrs. (A,E,I,M) whole body lateral views, (B,F,J,N) lateral view of head, (C,G,K,O) dorsal views, (D,H,L,P) magnified view of hindbrain rhombomeres. Control embryos (A-D, I-L) demonstrate *hoxb1a* staining (A-D) in rhombomere 4 and *krox-20* staining (I-L) in rhombomeres 3 and 5. Treated embryos (E-H, M-P) demonstrate normal *hoxb1a* staining (E-H) in r4 and normal *krox-20* staining (M-P) in r3 and r5. No

difference is seen between control and treated embryos in hindbrain staining patterns. r: rhombomere

Fig. 8. Retinoic Acid Responsive Transgenics. (A,C) whole body lateral views, (B,D) magnified lateral view of eye. Control embryos (A,B) demonstrate yellow fluorescence along the hindbrain and spinal cord and strong expression in the ventral retina. Treated embryos (C,D) demonstrate no change along the hindbrain and spinal cord, but a loss of expression in the ventral retina. FB(di): diencephalic portion of forebrain, FB(tel): telencepahlic portion of forebrain, MB: midbrain, MHB: mid-hindbrain boundary, HB: hindbrain, DR: dorsal retina, VR: ventral retina. RA responsive domain: arrow denotes beginning of the RA-sensitive domain in the hindbrain that extends down the spinal cord.

Fig. 9. Zn-8 immunohistochemistry. (A-C) frontal view of brain. Control (A) embryos demonstrate strong staining along the optic nerve and where it bifurcates into the retinal ganglion cells. The optic chiasm is also clearly seen. Treated embryos show moderate (B) and severe (C) loss of the signal in the optic nerve and chiasm. RGC: retinal ganglion cells.