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Exposure of *C. elegans* eggs to a glyphosate-containing herbicide leads to abnormal neuronal morphology

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Abstract

Recent data demonstrate that chronic exposure of *Caenorhabditis elegans* (*C. elegans*) to a high-use glyphosate-containing herbicide, Touchdown (TD), potentially damages the adult nervous system. It is unknown, however, whether unhatched worms exposed to TD during the egg stage show abnormal neurodevelopment post-hatching. Therefore, we investigated whether early treatment with TD leads to aberrant neuronal or neurite development in *C. elegans*. Studies were completed in three different worm strains with green fluorescent protein (GFP)-tagged neurons to facilitate visual neuronal assessment. Initially, eggs from *C. elegans* with all neurons tagged with GFP were chronically exposed to TD. Visual inspection suggested decreased neurite projections associated with ventral nerve cord neurons. Data analysis showed a statistically significant decrease in overall green pixel numbers at the fourth larval (L4) stage (*$p < 0.05$). We further investigated whether specific neuronal populations were preferentially vulnerable to TD by treating eggs from worms that had all dopaminergic (DAergic) or γ-aminobutyric acid (GABAergic) neurons tagged with GFP. As before, green pixel number associated with these discrete neuronal populations was analyzed at multiple larval stages. Data analysis indicated statistically significant decreases in pixel number associated with DAergic, but not GABAergic, neurons (***$p < 0.001$) at all larval stages. Finally, statistically significant decreases (at the first

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larval stage, L1) or increases (at the fourth larval stage, L4) in superoxide levels, a developmental signaling molecule, were detected (*p < 0.05). These data suggest that early exposure to TD may impair neuronal development, perhaps through superoxide perturbation. Since toxic insults during development may late render individuals more vulnerable to neurodegenerative diseases in adulthood, these studies provide some of the first evidence in this model organism that early exposure to TD may adversely affect the developing nervous system.

Keywords

Glyphosate-containing herbicides; neurodevelopment; Touchdown; DAergic neurons; *C. elegans* strain EG1285; *C. elegans* strain BZ555

1.0 Introduction

Concomitant with an increase in the use of genetically-modified agricultural crops (*e.g.*, cotton, corn, and soy beans), data suggest a parallel increase in pesticide use, particularly glyphosate-containing herbicides [27]. Touchdown® (TD; Syngenta) and Roundup® (Monsanto) are the two most commonly applied herbicides in this category [63]. Early studies evaluating the toxicity of glyphosate [58] indicated a relatively low acute toxicity for rats (oral LD_{50} = 2000 mg/kg) and mice (oral LD_{50} = 10,000 mg/kg). These experiments, however, seldom, if ever, investigated the toxicity of the whole commercial herbicide formulation (active compound with adjuvants). In the few comparison studies that have been published, results typically indicate that the toxicity of the formulation is greater than that of the active ingredient [5,50,59] or the putative surfactants (adjuvants) alone [41]. Specifically, studies have demonstrated mitochondrial inhibition in isolated liver mitochondria by Roundup®, but not by glyphosate alone [48]. Others also have shown differential genotoxicity between glyphosate and the formulation [50], although some question these findings [42,62]. Likewise, data from our lab suggest that *Caenorhabditis elegans* (*C. elegans*) exposed to various concentrations of a commercially available formation of TD results in apparent neurodegeneration, particularly of DAergic neurons [46]. As the use of this herbicide group has increased, so has the interest in the verifying whether differential toxicity actually exists between the commercial formulation and active ingredient [14,51].

Given recent research regarding the role that early (including pre- and perinatal) exposure to environmental toxicants may play in regulating gene expression [9,19,40], it is important to determine whether exposure to high-use pesticides might alter development. Furthermore, early toxic insults leading to subsequent disease phenotype expression is consistent with a "multiple-hit hypothesis" that may explain the "idiopathic" onset of various neurodegenerative diseases later in life [11]. For example, data already indicate that epigenetic changes following toxicant exposure early in life can lead to pathophysiology related to Alzheimer's, Parkinson's, or Hungtinton's disease [25], as well as autism and Rett syndrome [64]. Other epidemiological data strongly suggest a correlation between children diagnosed with attention deficit/hyperactivity disorder (ADHD) or attention deficit disorder (ADD), and exposure to pesticides [7,52]. Therefore, it is likely that early xenobiotic exposures may render individuals more susceptible to future toxic exposures.

In our previous studies, we showed that worms exposed to TD during the first and second larval stages (L1 and L2) showed DAergic neuron abnormalities as adults [45,46]. It is unknown, however, if *C. elegans* hatched from fertilized eggs that were exposed to TD would show similar neuronal abnormalities during the various developmental periods. Similar to other nematodes, *C. elegans* lay eggs that hatch and go through four larval stages (L1, L2, L3, and L4) that are partially designated by the end of a molt (L1 designates the larval stage after hatching; L2 is marked by the end of the first molt; L3 is marked by the end of the second molt; L4 is marked by the end of the third molt; young adult designation is used for the stage following the fourth molt). Neuronal development can be monitored at each different larval stage using various *C. elegans* strains with green fluorescent protein (GFP)-tagged neuronal populations. Thus, to test our hypothesis that early exposure of fertilized eggs to low concentrations of TD used for weed control [43,56] would abnormally affect neurodevelopment, we used fluorescence microscopy to follow neuronal maturation through all four larval stages to assess whether differences would be observed post-exposure. Finally, we also assayed superoxide levels at the various larval stages to see if changes in this signaling molecule could explain abnormalities in neuron development or growth.

2.0 Materials and Methods

2.1. Worm and Escherichia coli strains

N2 (wild-type), NW1229, BZ555, and EG1285 nematodes (Table 1), as well as NA22 *Escherichia coli (E. coli)* and OP50-1 *E. coli* were provided by the *Caenorhabditis* Genetics Center (CGC). NW1229 worms allowed assessment of all neurons in the worms, while BZ555 and EG1285 worms permitted visualization of DAergic or GABAergic neurons, respectively. In NW1229 worms (evIs111 [*F25B3.3::gfp* + dpy-20(+)]V), *F25B3.3* (*rgef-1*), a Ca^{2+}-regulated Ras nucleotide exchange factor, is fused with a green fluorescent protein gene (*gfp*). Reporter fusions of *rgef-1* are expressed in post-mitotic neurons (pan-neuronally) beginning during late embryogenesis [36]. BZ555 worms (egIs1 [*dat-1p::gfp*]), are genetically modified such that *dat-1p*, the plasma membrane dopamine transporter (DAT) promoter, is fused with *gfp,* which results in the green fluorescence of all DAergic neurons [24]. Finally, EG1285 worms (oxIs12 [*unc-47p::gfp* + lin-15(+)]) are genetically modified such that *unc-47p*, a promoter for the transmembrane vesicular γ-aminobutyric acid (GABA) transporter (GAT), is fused with *gfp*, leading to expression of green fluorescent protein in all GABAergic neurons [37].

2.2 Synchronization

In order to isolate a sufficient number of worms, adult *C. elegans* were grown on 8P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 0.5 mM potassium phosphate buffer [pH 6], 0.013 mM cholesterol [in 95% ethanol], 1 mM $MgSO_4$) with a lawn of NA22 *E coli* (grown in 16 g tryptone/L, 10 g yeast extract/L, 85.5 mM NaCl) until gravid. Per standard protocols, NA22 bacteria was used on these plates because their rapid growth and reproduction can sustain large numbers of worms [55]. Worms were then washed off of the plates and into tubes to facilitate exposure to a bleaching solution (0.55% NaOCl, 0.5 mM NaOH), which allows or promotes the release eggs from the gravid adult.

The egg/bleach mixture was diluted and washed with M9 solution (20 mM KH_2PO_4, 40 mM Na_2HPO_4, 68 mM NaCl), exposed to 30% sucrose, and then centrifuged to separate eggs from debris.

2.3 Treatment of Isolated C. elegans' Eggs

Eggs obtained from synchronization were treated with the indicated concentrations of glyphosate as the commercial formulation of TD one hour post-synchronization. Eggs in the respective treatment solution were then placed on fresh NGM/OP50-1 plates at 20°C for an additional 25 hours, providing a chronic exposure prior to hatching. Once the worms from the treated eggs had hatched, they were removed from plates and washed an additional 3X with dH_2O to remove residual TD. Pictures were taken at varying times post-incubation of 3–5 worms from each treatment group using a digital camera attached to a fluorescence microscope: one hour (L1), nine hours (L2), 21 hours (L3), or 46 hours (L4). Since the focus of this study was to determine if treatment of *C. elegans'* eggs could lead to neuronal changes during development, time points after L4 (young adult and adult) were not assessed.

2.4 Viability and Fecundity Studies

Eggs from wild-type (N2) worms were initially treated with varying concentrations of glyphosate (as TD) for 30 min in microcentrifuge tubes. Following this 30 min incubation, the number of eggs/µL solution was determined so that a total of 50 eggs would be consistently added to seeded NGM/OP50-1 plates for each replication. Twenty-four hour post-hatching, the number of moving worms was counted on each plate to determine the number of live animals. For fecundity studies, 15 offspring were picked from the NGM/OP50-1 plates used for egg studies, and were transferred to fresh NGM plates (five worms/plate) with an OP50-1 lawn. These nematodes were allowed to grow (in the absence of additional TD) and reproduce. The number of live offspring laid within a 24-h period was counted and normalized to the number of live offspring from control hermaphrodites.

2.5 Microscopy

Photomicrographs were taken of three to five worms on 4% agarose pads with a fluorescence microscope (Leitz & Wetzlar, Halco Instruments, Inc) equipped with a 50W AC mercury source lamp (E Leitz, Rockleigh, NJ) and a 40X objective (Leitz & Wetzlar, Halco Instruments, Inc.). A digital camera (Micrometrics, Miles Co Scientific, Princeton, MN) operated by Micrometrics software (Micrometrics SE Premium) was attached to the microscope to obtain images. The images were standardized by programming software to ensure that pictures were obtained with identical levels of gamma, gain, exposure time, saturation, and color gain. Photomicrographs were opened with Adobe Photoshop and the total number of green pixels was recorded for further data analysis.

2.6 Dihydroethidium (DHE) Detection

Eggs obtained from synchronizations of N2 worms were treated with 5.5% glyphosate (as TD) for 30 min, and then placed on fresh NGM/OP50-1 plates and incubated at 20°C for 24 h. After incubation, worms were removed from plates, and then washed 3X with dH_2O to remove residual TD. Worms at various life stages were subsequently exposed to 500 µM

dihydroethidium (DHE; EMD Millipore, Billerica, MA), which forms a red fluorescent product when it reacts with superoxide, for three hours at 20°C. At the conclusion of the DHE incubation, control and treated worms from each life stage were visualized by a BenchTop 2 UV-Transilluminator light box (UVP, Upland, CA) at $\lambda = 302$ nm. Photomicrographs were analyzed using Adobe Photoshop to determine the number and intensity of red pixels, which is indicative of DHE activation.

2.7 Data Analysis

Data analysis was performed using GraphPad Prism (v5.03 for Windows, GraphPad Software, San Diego, CA). For each synchronization, experiments were completed a minimum of three times (n ≥ 3) with analysis of three to five worms, resulting in a minimum of twelve worms for each data point. TD concentrations are presented as percent active ingredient (% glyphosate) in order to facilitate comparisons with other glyphosate-containing herbicides [45,46]. Differences in pixel number were determined by one-way analysis of variance (ANOVA), followed by a *post-hoc* Bonferroni test. Data were considered to be statistically significant if $*p < 0.05$.

3.0 Results

3.1 Treated Eggs Show Decreased Offspring Viability

Eggs from wild-type (N2) worms were chronically (24 h) treated with varying concentrations of TD (normalized to percent glyphosate) and allowed to hatch. Twenty-four hours post- treatment, the number of hatched, live nematodes per plate was counted (Figure 1). Analysis of data, normalized to the number of eggs successfully hatched by control (CN) worms (Figure 1a), indicated a statistically significant, dose-dependent decrease in viability of larva ($***p < 0.001$). Furthermore, surviving hermaphrodites from eggs chronically treated with TD laid statistically significantly fewer offspring ($**p < 0.01$ for 5.5% glyphosate as TD, or $***p < 0.001$ for 9.8% glyphosate as TD) than CN worms (Figure 1b). It is important to note that brood size, which refers to the total number of eggs a *C. elegans* lays in a lifespan, was not determined. The data presented here represents the number of eggs laid within a 24-h period by adult animals that were treated as embryos. This number is consistent with the results of others who have measured the number of eggs at the end of 24 h [28,29], but less than the average of 300 eggs often reported for brood size over a life-time [31]. Taken together, these initial data suggest that early-life exposure of *C. elegans* to TD may adversely affect common reproductive parameters in these worms.

3.2 Worms from Treated Pan-Neuronal::GFP[1] (NW1229) Eggs Show Neurodegeneration

While a decrease in the number of viable offspring may provide important clues regarding teratogenicity or infant death, it provides little evidence that early toxicant exposure could lead to greater vulnerability to environmental insults later in life. Thus, we extended these studies to include *C. elegans* with GFP reporter constructs to follow neuron morphology

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[1]The authors have taken the liberty to modify the generally accepted abbreviations for *gene::gfp* or protein::GFP in such a way as to provide information for the neuronal populations tagged with GFP. This is meant to provide a short-hand for readers who may be unfamiliar with the abbreviations while concurrently providing information about the actual worm strain. For detailed *gene::gfp* information, please see the **Section 2.1** in Materials and Methods.

throughout each larval stage. In order to determine whether exposure of nematode eggs to TD resulted in morphologic changes to neuronal populations, we initially treated fertilized eggs from the pan-neuronal tagged NW1229 strain (Table 1) with this herbicide. A statistically significant decrease (***$p < 0.001$) in the total number of pixels throughout the entire worm was observed only at the L4 stage (Figure 2a).

In order to determine if a one general region contributed more (or less) to this decrease, the nerve ring was assessed separately from the ventral nerve cord. This separation also allowed for a gross assessment of specific neuronal populations since the ventral nerve cord is predominantly composed of cholinergic and GABAergic neurons [34], and the nerve ring is largely comprised of DAergic neurons [13]. When the ventral nerve cord was analyzed separately, a statistically significant decrease (*$p < 0.05$) in pixel number was observed at the L4 stage (Figure 2b). Visual inspection of the ventral nerve cord suggested the decreased pixel number resulted from a loss of discrete and defined expression of GFP, leading to an overall reduction of neuron size (yellow arrow), and an apparent loss of neuronal connectivity (blue arrows) when compared to CN worms (Figures 2c & 2d). Separate pixel analysis of the nerve ring indicated no statistically significant difference between CN and treated worms at any of the assessed larval stages (data not shown).

3.3 Worms from Treated vGAT::GFP (EG1285) Eggs Show Neurodegeneration

To better characterize the neuronal populations adversely affected in the ventral nerve cord, we next used the EG1285 strain, in which all GABAergic neurons are tagged with GFP. We determined that using this strain would allow for better assessment of this neuronal population since there are only 26 GABAergic neurons [34], as compared to the 120 cholinergic neurons [49], in *C. elegans*. When photomicrographs of CN worms were compared to those from treated worms, no statistically significant change in pixel number was detected across development from L1–L4 (Figure 3a). Visual inspection supported the quantitative analysis. These data strongly suggest that the decreased pixel number observed in the pan-neuronal-GFP worms (Figure 2) was due changes associated with cholinergic, rather than GABAergic, neurons.

Worms lacking the ability to synthesize or package acetylcholine are often smaller than their wild-type counterparts, and they are usually found on plates in a coiled pattern instead of a sinusoidal line [49]. This distinctive coiled pattern was observed via light microscopy for many of the treated worms. Conversely, an increased head angle, the phenotypic hallmark of loss of GABA neurons [34], was not observed in treated worms. These observations also support the hypothesis that decreased GFP in the ventral nerve cord is likely due to damage to the cholinergic neurons.

When treatment concentrations, however, were increased to 5.5% or 9.8% glyphosate (as TD), a statistically significant decrease in pixel number (*$p < 0.05$) was found in EG1285 larva only at the L4 stage (Figure 3b). Visual inspection of worms in the higher treatment concentration groups did have the characteristic 'hooked' head, indicative of the abnormally sharp head angle associated with GABAergic neuronal damage, when compared to CN worms in the same life stage (Figures 3c and 3d). Many treated worms also showed the coiled body orientation as seen before in the lower concentrations. Both of these informal

observations are consistent with degeneration of acetylcholine and GABA neurons at higher TD concentrations.

3.4 Worms from Treated dat-1::GFP (BZ555) Eggs Show Neurodegeneration

Although no statistically significant decreases in pixel number associated with neurons in the nerve ring were observed in the pan-neuronal-GFP strain, we were still interested in a greater characterization of DAergic neurons in this region. This is also particularly important since many neurological disorders that appear later in life are known to result from perturbations in dopamine levels or changes in DAergic neuronal viability [1,21,23]. Thus, we next treated eggs from the BZ555 strain (all DAergic neurons tagged with GFP) with TD. Interestingly, beginning with L1-staged worms there was a statistically significant (***$p < 0.001$) decrease in the number of pixels associated with the DAergic neurons in treated worms compared to CN (Figure 4a). This decrease remained consistent through all four larval stages, with the greatest difference observed at L4. Visual inspection of photomicrographs (Figures 4b–4e) suggested that the decrease was at least partially due to loss of GFP associated with the neuronal processes or neurites (blue arrows) rather than morphologic changes of the somas.

3.5 Worms from Treated Eggs Show Changes in Superoxide Production

During development, multiple reactive oxygen species (ROS), particularly superoxide ($^{\bullet}O_2$) [22], act as important signaling molecules. Because changes in the levels of this molecule could potentially alter offspring viability or neurodevelopment, we treated fertilized eggs from N2 (wild-type) *C. elegans* with TD, followed by incubation with dihydroethidium (DHE), which reacts with $^{\bullet}O_2$ to produce a red fluorescent compound. Since neurodegeneration was apparent in both the BZ555 and EG1285 strains starting at a mid-range herbicide concentration, we used this amount in the DHE studies. Following treatment with 5.5% glyphosate as TD, worms were exposed to DHE to determine whether changes in $^{\bullet}O_2$ levels could be detected. There was a statistically significant decrease (*$p < 0.05$) in DHE fluorescence in the TD-treated group compared to CN worms at the L1 stage (Figure 5a), but a statistically significant increase (***$p < 0.001$) in DHE fluorescence at L4 (Figure 5b). These data suggested that DHE regulation is impaired during two critical, but distinct, larval stages.

4. Discussion

With the increased use of glyphosate-containing herbicides, such as Touchdown® (TD; Syngenta) and Roundup® (Monsanto), many current studies are finally beginning to examine the potential toxicity of the commercially available formulations, and not just the active ingredient or the putative surfactants and associated vehicle(s) [14,32,38]. Using *C. elegans* as a model organism, our lab has focused on the potential neurotoxicity of TD, as obtainable by industrial and household pesticide applicators [45,46]. In the present study, we wanted to test the hypothesis that *C. elegans* treated with TD during their egg stage could lead to abnormal neurodevelopment. Initially, we wanted to determine whether this exposure protocol would lead to overall and gross adverse effects on offspring viability.

Following treatment of the worm eggs with varying concentrations of TD, we observed a decrease in fecundity in a 24-h period. This decreased offspring fecundity was even observed in the absence of any additional exposure of TD to the hatched worms (Figure 1). Although there was also a reduction in the number of eggs laid, we focused our attention on potential neurodevelopmental, rather than reproductive, toxicity. As such, we were interested in using these worms to further determine whether *C. elegans* exposed to TD while in their egg stage would have morphologic changes associated with discrete neuronal populations. It is important to address this question because a multiple-hit hypothesis for neurodegenerative diseases predicts that environmental insults early in development, particularly during critical periods, could render offspring more vulnerable to additional insults later in life [11,57].

In order to determine whether damage occurred to the nervous system, we exposed eggs from pan-neuronal GFP-tagged worms (NW1229) to TD. Our initial screen, using visual inspection of neurons and pixel number analysis, indicated that treated worms were similar to CN worms until the L4 stage. At this point in development a statistically significant decrease (*$p < 0.05$) in the number of green pixels associated with neurons were observed (Figure 2). These results paralleled the loss of neurites and abnormal soma morphology observed using light and fluorescence microscopy. While these data suggested that the developing nervous system of *C. elegans* might be vulnerable to TD exposure, it did not provide information as to whether specific neuronal populations were differentially vulnerable. In our previous studies assessing worms treated with TD at the L1/L2 stage, we observed that GABAergic and DAergic neurons were among the first to show morphological changes associated with loss of pixel numbers [45,46]. Since it is difficult to clearly identify these two populations in the pan-neuronal::GFP strain, we continued our studies in GABA neurons::GFP worms (EG1285) and DA neurons::GFP worms (BZ555).

Although GABAergic neurons are not typically associated with signs or symptoms of major neurodegenerative or neurodevelopmental diseases, recent research has demonstrated their importance in the early symptomology of Parkinson's disease [8,17,35] and Alzheimer's disease [39,47,54]. In our herbicide model, however, these GABAergic ventral nerve cord neurons do not appear to contribute significantly (Figure 3) to the loss of green pixels observed in the pan-neuronal::GFP studies (Figure 2). Rather, behavioral data, as well as absence of statistically significant decreased pixel number in the EG1285 worms, suggest that TD exposure in *C. elegans* results in the loss of cholinergic neurons and/or their processes at the lower TD concentrations. Because various ventral cholinergic neurons are responsible for innervating muscles associated with egg laying [2], loss or dysfunction of these neurons could potentially explain the decreased number of eggs observed in our initial viability studies. In the absence of this innervation, fewer eggs are laid [61]. Thus, loss or dysfunction of choline neurons could also potentially explain the decreased number of eggs observed in our initial viability studies (Figure 1). In humans, however, the cholinergic neurons of the pedunculopontine nucleus complex and thalamus, and the interneurons of the striatum are thought to contribute to some of the postural instability observed in PD patients [8,44]. Thus, changes in cholinergic neurons would be expected to exacerbate signs and symptoms associated with degeneration of the DAergic system.

In addition to PD, however, many neurodegenerative and behavioral diseases are associated with changes in DA levels. Thus, we also took a closer look at the extent of DAergic neurodegeneration in *C. elegans* following exposure to TD using the DAergic neurons::GFP (BZ555) worm strain. Visual confirmation of decreased neuron size throughout all larval stages (L1–L4) was corroborated by green pixel analysis (Figure 4). We also noted that these worms did not move in the normal sinusoidal pattern consistent in control worms. Rather, they tended to move into a 'comma' pattern, and lacked high-angle turns in feeding, both of which are consistent with behavior associated with worms lacking DA or DA receptors [13]. The damage to the DAergic neurons was much more extensive than that observed in other populations we assessed. Furthermore, the gaps between total pixel number and associated changes in neuronal morphology were greater at the L4 stage than at the earlier larval stages, which suggest that the treated worms were unable to compensate for the damage associated with the early TD exposure. While the results should be repeated in higher model organisms, our data from *C. elegans* do suggest that early exposure to TD could render a developing organism more vulnerable to DAergic insults later in life.

Finally, in order to identify a potential mechanism to explain our results, we treated N2 eggs with the mid-range concentration of TD that decreased GABAergic neuronal pixel numbers early in development. In particular, we wanted to determine whether we could detect changes in superoxide production, which can lead to hydrogen peroxide formation. We assayed for changes in this particular ROS because it and hydrogen peroxide regulate various aspects of signaling and cellular differentiation [4,20,60]. At the L1 stage we detected a statistically significant decrease (*$p < 0.05$) in superoxide levels in treated worms compared to CN (Figure 5). This could result from an inhibition in superoxide production, an increased turnover to hydrogen peroxide, or activation/upregulation of antioxidant proteins such as superoxide dismutase. This was beyond the scope of our study, but such mechanisms are consistent with similar endpoints reported following exposure to other pesticides [15,16] By the L4 stage, the decrease completely reversed, and a statistically significant increase (***$p < 0.001$) in superoxide was detected. In this case, it could be that any protection afforded by upregulation in antioxidant proteins had been overwhelmed by this later larval stage. Although the TD concentration in these studies was higher than the 2.7% glyphosate used on the transgenic worm strains, it is still well within the manufacturer recommended levels [6,43] for spot spraying weeds, and has been used in other studies from this lab [45,46].

Hermaphroditic *C. elegans* only have eight DAergic neurons (CEPDL, CEPDR, CEPVL, CEPVR, ADEL, ADER, PDEL, PDER) in their nervous system [13]. Four of these (ADEL/R, and PDEL/R) develop post-embryonically during the second larval (L2) stage [3]. Furthermore, the polarity of the PDE cells are under the control of the Wnt pathway, as is the general position of the nerve ring [53], where many of the DAergic neurons are located [13]. It is also known that changes in the levels of ROS, particularly superoxide and hydrogen peroxide, can regulate the ability of Wnt-related transcription factors to translocate to the nucleus [18]. The other DAergic neurons (CEPDL/R and CEPVL/R), as well as the ADEL/R neurons, all originate from embryonic precursor cells identified as AB, which are formed during the first cell division [26]. Thus, we suggest that the changes in ROS status documented in our studies could provide a mechanistic explanation as to why the DAergic

neurons were more vulnerable to changes when compared to other neuronal populations (Figure 6).

Although *C. elegans* are removed from humans on the evolutionary ladder, their nervous system shares a remarkable similarity to that of humans [10,12]. These include important aspects such as homologous and orthologous genes for neurotransmitter receptors, synthesis enzymes, and vesicular and membrane transporters [30]. Furthermore, while the eggshell protecting the early embryonic development of *C. elegans* does not have the same chemical composition as the human placenta [33], its function is roughly that of its mammalian counterpart: to protect the growing larva/fetus from environmental toxicants. As such, we suggest that our egg-treatment studies serve as a good first-approximation to model *in utero* exposure to agrochemicals. Although these results should be replicated in higher model organisms, our data suggests initial areas of interest for further studies. In conclusion, we have demonstrated that eggs from *C. elegans* exposed to TD results in larva with abnormal neuronal cell bodies and/or neurites, and that these developmental abnormalities could be regulated by early larval-stage alterations in superoxide concentrations. These data may also shed light on why prenatal exposure to various pesticides could lead to greater vulnerability to neurodevelopmental or neurodegenerative diseases later in life.

Acknowledgments

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Highlights

- *C. elegans* hatched from eggs exposed to Touchdown have decreased fecundity compared to non-treated worms;

- General changes in neurodevelopment are not observed until the fourth larval (L4) stage;

- GABAergic neurons are less vulnerable than DAergic neurons to exposure to Touchdown;

- DAergic neurons show altered neurodevelopment as early as the first larval (L1) stage;

- Abnormal reactive oxygen species levels may be responsible for the observed toxicity.

a)
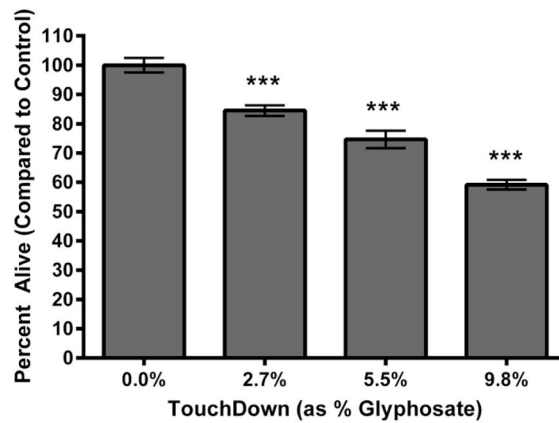
b)
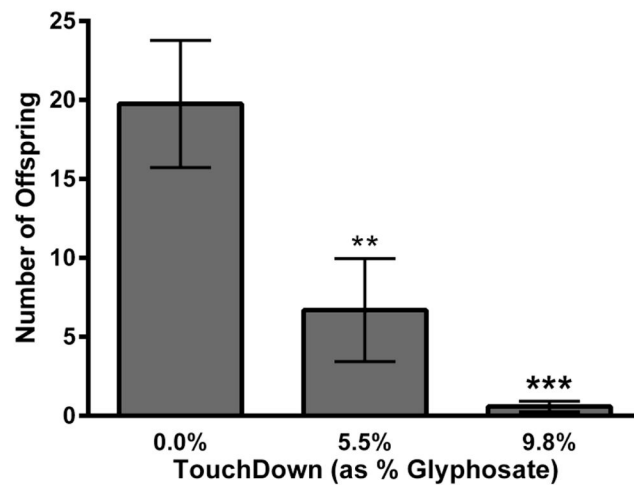
Figure 1.
Decreased larval viability following chronic treatment with TD. (**a**) Eggs from wildtype (N2) worms treated chronically with varying concentrations of TD (as percent glyphosate) showed a statistically significant and dose-dependent decrease in viable offspring compared to CN worms. (**b**) Hermaphrodites that successfully hatched from treatment groups in Figure 1a were allowed to grow and reproduce. Total number of live offspring over a 24-h period was statistically significantly decreased in a dose-dependent manner compared to CN. Data are presented as mean ± SEM; **p < 0.01, ***p < 0.001.

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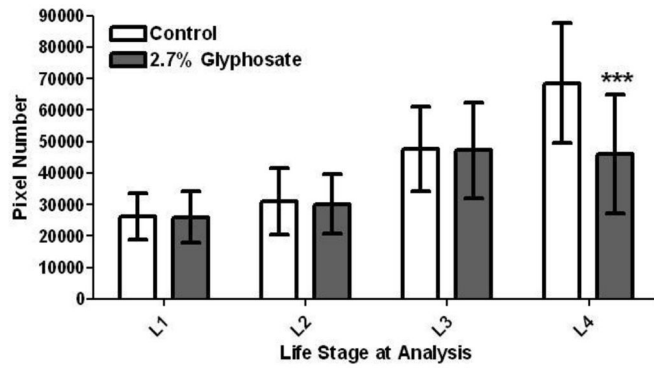
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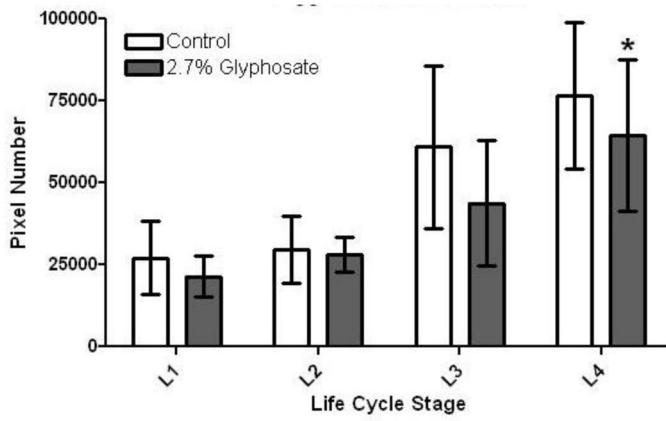
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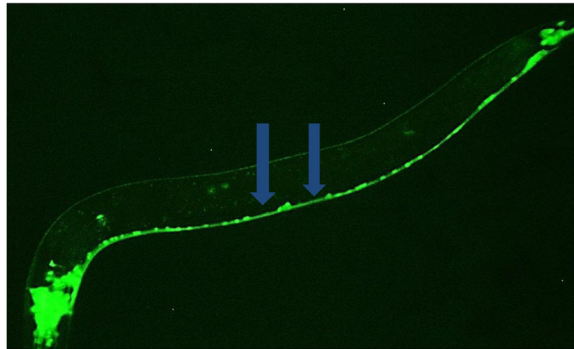
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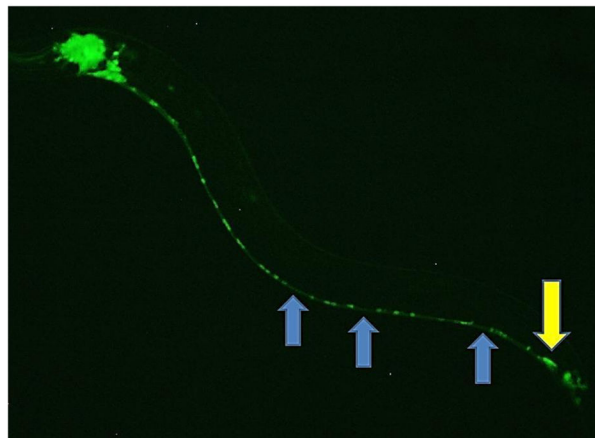
Figure 2.
Decreased neuron-associated green fluorescence following chronic TD exposure. Following treatment of eggs from the pan-neuronal::GFP (NW1229) strain with varying concentrations of TD (as percent glyphosate), number of green pixels was determined (see **Section 2.5**). Data from analyzing pixel number associated with either the entire worm (**a**) or only the ventral nerve cord (**b**) are presented as mean pixel number ± SD. *p < 0.05, ***p < 0.001 compared to CN. Photomicrographs of a representative control (**c**) or treated (**d**) worm at the L4 stage show abnormal regions in the nervous system. The photomicrograph of the CN worm demonstrates nerve ring neurons in close proximity to the cuticle, and nerve cord neurons with their associated neurites. The representative photomicrograph of an age-matched treated worm shows gaps or diminished connectivity of neurites (blue arrows) and apparent morphological changes in nerve cord neurons (yellow arrow).

a)
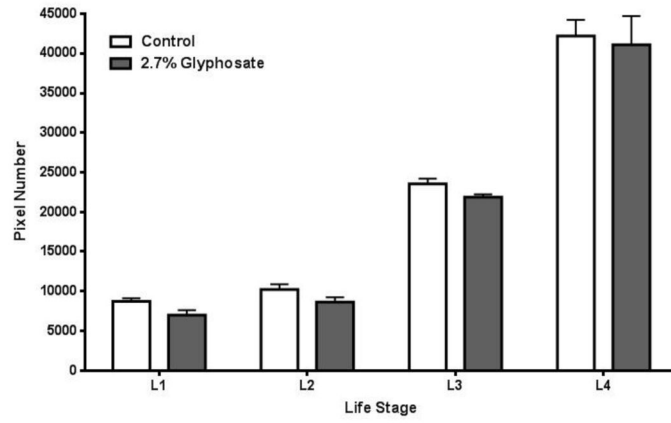
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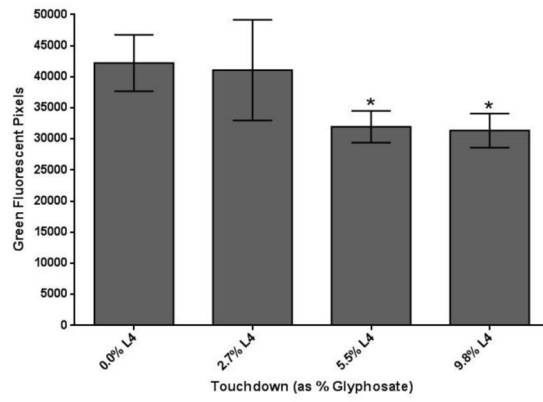
c)
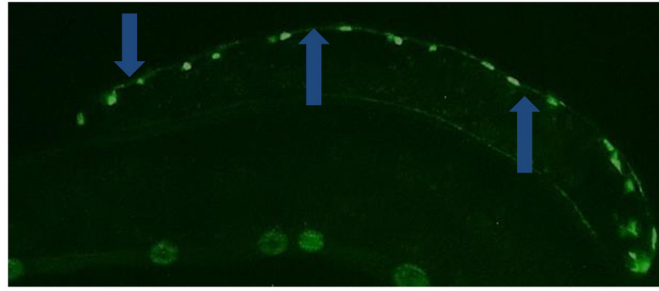
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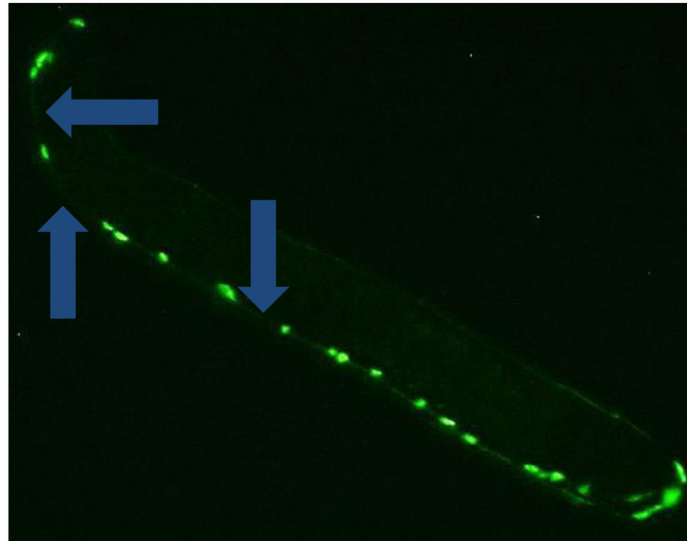
Figure 3.
Chronic TD treatment of eggs from GABA::GFP (EG1285) worms at mid- and high-TD concentrations leads to decreased green pixel numbers at the L4 stage. (**a**) Eggs from EG1285 worms treated chronically with a low concentration of TD (as percent glyphosate) did not lead to decreased pixel numbers or apparent morphological changes in worms at any life stages. (**b**) When TD concentrations (as percent glyphosate) were increased, however, a statistically significant decrease in pixel number was observed compared to CN worms. Data are presented as mean ± SEM; *p < 0.05. Visual comparison of CN worms (**c**) to those treated the highest concentration of TD (**d**) indicated that loss of fluorescence was likely due to neurite degeneration (blue arrows).
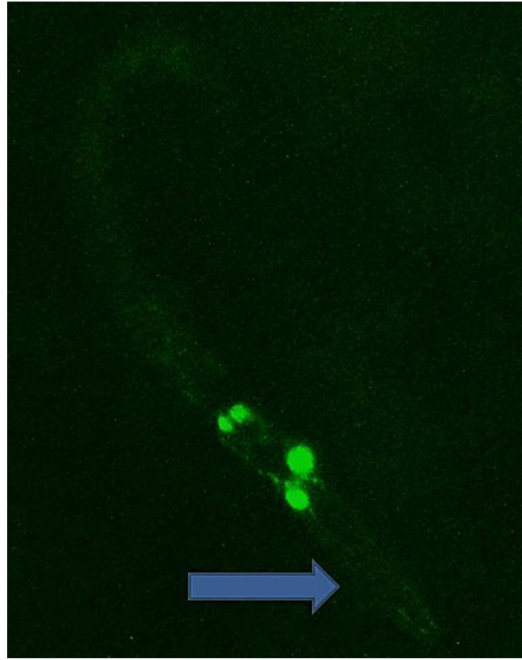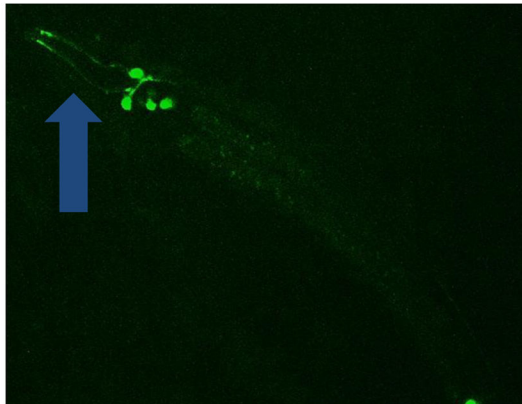
a)
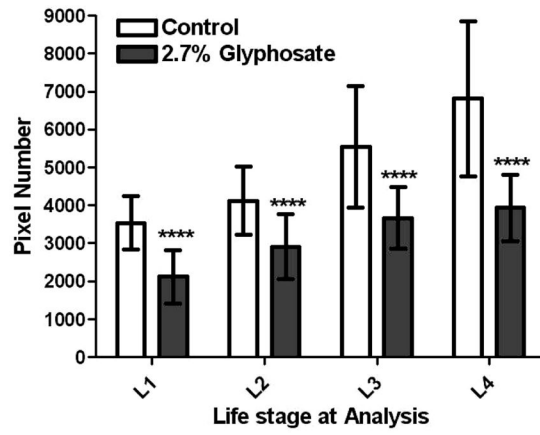
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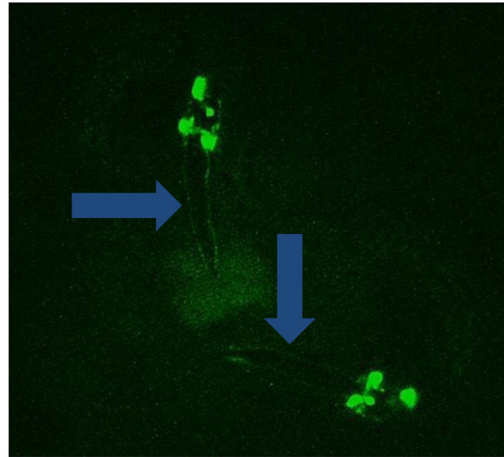
c)

d)

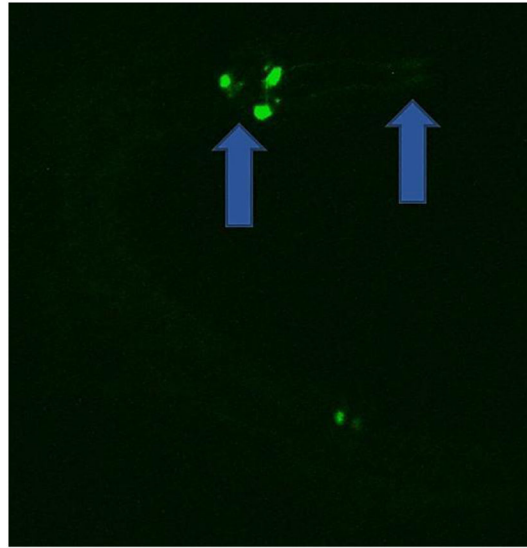**e)**

Figure 4.
Chronic TD treatment of eggs from DA::GFP (BZ555) worms leads to decreased green pixel number associated with DAergic neurons and their neurites. Following treatment with TD (as 2.7% glyphosate), number of green pixels was determined as described in **Section 2.5**. (**a**) Data analysis of pixel numbers from DAergic neurons. Data are presented as mean pixel number ± SD. ***p < 0.001 compared to CN. Photomicrographs of representative control (**b**) or treated (**c**) worms at the L1 stage, and control (**d**) or treated (**e**) worms at the L4 stage. Photomicrographs of control worms depict DAergic neurons and their projections, which are clearly visible. This is in contrast to the same projections in treated worms (blue arrows).

a)
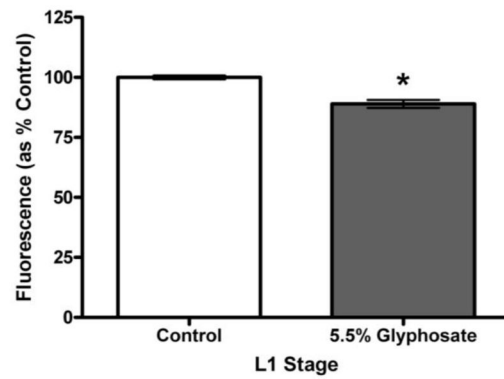
b)
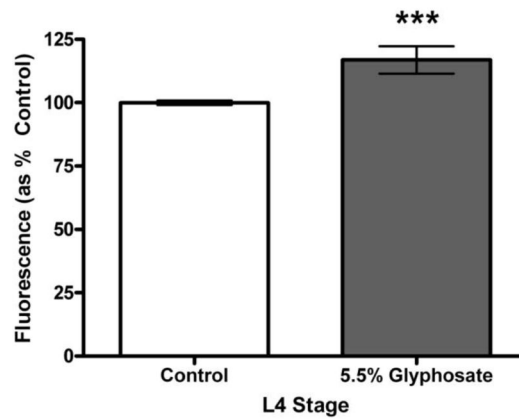
Figure 5.
Altered superoxide production following chronic treatment of eggs with TD. Eggs from N2 worms treated chronically with varying concentrations of TD (as percent glyphosate) showed a statistically significant decrease in superoxide production at the L1 stage (**a**), but a statistically significant increase at L4 (**b**) compared to control worms. Data are presented as mean ± SEM; *p < 0.05, ***p < 0.001.
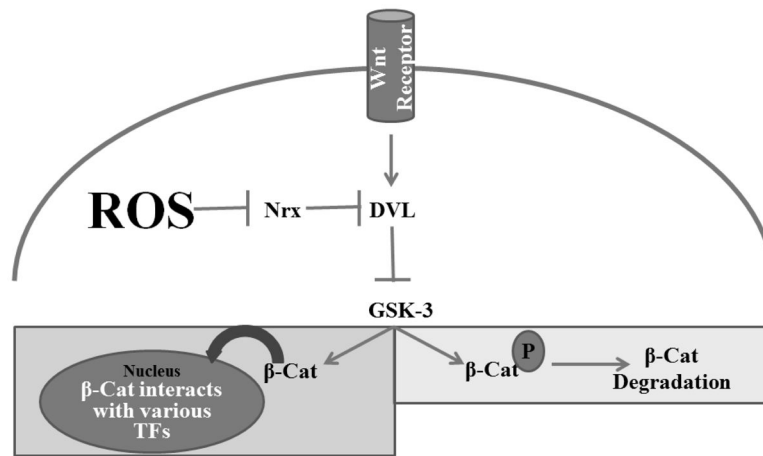
Figure 6.
Simplified Wnt/ β-catenin pathway in *C. elegans* showing how altered ROS production could disrupt normal transcription of genes associated with neurodevelopment of DAergic neurons in *C. elegans*. In the absence of Wnt ligands, glycogen synththase kinase-3 (GSK-3) will phosphorylate β-catenin (β-Cat), leading to its degradation (light gray box). When Wnt ligands bind to Wnt receptors, GSK-3 is prevented from phosphorylating β-Cat, allowing it to translocate to the nucleus where it can activate various transcription factors (TFs) in the nucleus (dark gray box). The abnormal presence of reactive oxygen species (ROS) can inhibit nucleoredoxins (of which R05H5.3 and C32D5.8 are orthologs in *C. elegans*), which leads to the phosphorylation of β-Cat by GSK-3.

Table 1

Strain Designation	Tagged Neuronal Population	Gene Promoter	Protein
N2	None	None—Wild-type	None—Wild-type
NW1229	All neurons	*regf-1*	Ca^{2+}-regulated Ras nucleotide exchange factor
BZ555	Dopamine	*dat-1p*	Dopamine transporter
EG1285	GABA	*unc-47p*	Vesicular GABA transporter