OXFORD

SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

*TOXICOLOGICAL SCIENCES*, 157(1), 2017, 129–140

doi: 10.1093/toxsci/kfx020
Advance Access Publication Date: January 25, 2017
Research article

# Embryonic Exposure to the Environmental Neurotoxin BMAA Negatively Impacts Early Neuronal Development and Progression of Neurodegeneration in the Sod1-G93R Zebrafish Model of Amyotrophic Lateral Sclerosis

Samantha Powers,*,† Samantha Kwok,* Emily Lovejoy,* Tom Lavin,* and Roger B. Sher*,‡,1

*Department of Molecular and Biomedical Sciences, University of Maine, Orono, Maine 04469; †Department of Neuroscience, The Ohio State University, Columbus, Ohio 43210; and ‡Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, New York 11794

1To whom correspondence should be addressed. Fax: 631-632-6661. E-mail: roger.sher@stonybrook.edu.



## ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder leading to progressive paralysis and death within 2–5 years after diagnosis. Sporadic cases (SALS) comprise approximately 90% of cases with the remaining 10% familial (FALS) caused by mutations in approximately 27 genes. The vast heterogeneity seen in age and location of disease onset, rate of progression, and duration of disease has been linked with genetic and environmental influences in both SALS and FALS cases. Increased ALS incidence clusters in Guam, southern France, and Maryland have been linked with exposure to Beta-methylamino-L-alanine (BMAA), a nonproteinogenic amino acid produced by cyanobacteria, dinoflaggelates, and diatoms. We embryonically exposed zebrafish, *Danio rerio*, (transgenically overexpressing a FALS-causing SOD1-G93R mutation) to BMAA to investigate early motor neuron outgrowth in larvae and endurance and fatigability in 5-month adults. SOD1-G93R zebrafish showed decreased embryonic nerve length with increased BMAA dose, a phenotypic change mirrored in 5-month performance measures of weaker swimming and increased fatigability. In contrast, transgenic fish overexpressing wild-type SOD1 were resistant to phenotypic changes, indicating a potential neuroprotective function of healthy SOD1. We show that the etiology of genetic ALS animal models can be influenced by environmental exposures, and that embryonic toxin exposures can result in changes to both early and adult measures. We demonstrate that zebrafish can be a robust model for investigating causes of ALS heterogeneity. Establishing these links between developmental and adult ALS-like symptoms in the zebrafish increases the power of this model for toxicological and drug screens.

**Key words:** amyotrophic lateral sclerosis; SOD1; beta-methylamino-L-alanine; BMAA; harmful algal blooms; climate change.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease with no identified mechanism, effective treatment, or cure. It leads to a progressive loss of both upper (brain) and lower (spinal cord) motoneurons leading to weakness, spasticity, hyperreflexia, muscle atrophy, paralysis, and death (Bruijn *et al.*, 2004; Pasinelli and Brown, 2006). The mean disease duration after diagnosis is 4 years (Pasinelli *et al.*, 2006). It is the third most common neurodegenerative disease and the most common adult onset neurodegenerative disease (Martin, 2011), with a prevalence of 4–8 cases out of 100 000

(Andersen, 2006) although this varies by ethnicity and geography (Caller *et al.*, 2012). There are approximately 30 000 U.S. Americans living with the disease and 5000 new cases diagnosed in the United States each year (Pasinelli *et al.*, 2006). The only FDA approved drug used to treat ALS is Riluzole, which only extends patient life by a few months (Bensimon *et al.*, 1994). However, many patients chose not to use Riluzole, because it does not improve ALS symptoms but leads to many adverse effects including nausea, vomiting, weakness, tachycardia, somnolence, headache, dizziness, vertigo, pain, parasthesia, and alterations in liver function tests (Stewart *et al.*, 2001).

Genetically, approximately 10% of ALS cases are familial ALS (FALS) (Kiernan *et al.*, 2011). The genetics of FALS cases are very complex, with hundreds of different mutations on 27 Mendelian genes linked to ALS in human patients (Guerreiro *et al.*, 2015). The other 90% of cases, which cannot be traced to a heritable mechanism, are described as sporadic (SALS) (Brooks, 1994), and there is no clinically distinguishing characteristic between the 2 forms of the disease except an earlier mean age of onset for FALS patients with high penetrance mutations (Andersen and Al-Chalabi, 2011; Testa *et al.*, 2004). Phenotypically, ALS cases differ in the location of onset, rate and pattern of progression, the relative levels of degeneration among upper and lower motoneurons (Simon *et al.*, 2014), age of onset, and disease duration (Swinnen and Robberecht, 2014). This huge phenotypic variation in both SALS and FALS indicates that gene–environmental interactions during development and growth play a role in the etiology of ALS (Bradley *et al.*, 2013) and the determination of phenotypic variations (Trojsi *et al.*, 2013).

A suite of environmental neurotoxicants has been associated with the development of ALS, with epidemiological, clinical, and experimental evidence indicating that early developmental exposures to neurotoxins can have consequences for neurotoxicity later in life (Fox *et al.*, 2012). A recent study by Roy *et al.* (2012) showed that brief embryonic exposure of zebrafish to strychnine results in adult neurobehavioral defects in swimming speed and diving behaviors. Alterations in serum microRNAs patterns in SALS parallel those seen in young asymptomatic FALS mutation carriers and in clinical FALS patients, independent of the specific genetic defect itself (Freischmidt *et al.*, 2014), part of an increasing body of evidence that the development of ALS is a multi-step process, similar in etiology to cancer, where a genetic defect can lead to several outcomes depending on subsequent environmental triggers (Al-Chalabi *et al.*, 2014). This need for multiple steps in disease progression would account for adult onset (even among patients with SALS), pleiotropy of some ALS genes, and spread of symptoms from a focal point that reaches the final level of cumulative steps (Al-Chalabi *et al.*, 2014). The current understanding is that ALS is part of a spectrum with other neurodegenerative diseases, and that they are the product of the combination of genetics, environmental factors, and metabolism (Zufiria *et al.*, 2016).

Harmful Cyanobacterial Blooms (HABs) are increasing worldwide due to changing climate (Erdner *et al.*, 2008; Hudnell, 2008; Merel *et al.*, 2013), and although acute toxic reactions to HABs are well known (nausea, vomiting, skin irritation, hepatotoxicity, neurotoxicity) (Hilborn *et al.*, 2014), the long-term impacts to exposed individuals are still undetermined, as are the potential interactions between genetic susceptibility and exposure. Exposure to repeated cyanobacterial blooms has been associated with an increased risk of ALS in New Hampshire (10–25-fold increase) (Caller *et al.*, 2009; Torbick *et al.*, 2014) and Southern France (Masseret *et al.*, 2013). One cyanobacterial neurotoxin associated with the development of ALS is Beta-methylamino-L-alanine (BMAA), a nonproteinogenic amino acid produced by cyanobacteria, diatoms (Jiang *et al.*, 2014), and dinoflaggelates (Lage *et al.*, 2014). In Guam in the 1940s–1950s, the native Chamorro people exhibited a very high incidence (50–100× the world average ALS incidence) of ALS/Parkinsonism dementia complex, an ALS-Plus Syndrome (Koerner, 1952). Cycad seeds in Guam were a dietary staple for the Chamorro population and for wild pigs and flying foxes, which in turn made up a large portion of the Chamorro diet. It was later determined that the BMAA was produced by cyanobacteria living in symbiosis with the cycad roots, and translocated to the seed. Biomagnification of the BMAA in the cycad seeds and animals feeding on them led to a biomagnification of BMAA in the Chamorro people (Banack and Cox, 2003), with BMAA exposure being the only statistical correlate to their extremely high incidence of ALS (Reed *et al.*, 1987). Latency periods between Guam residency and ALS symptom development as long as 34 years have been seen among Chamorro immigrants (Garruto *et al.*, 1980), indicating that early exposures to BMAA may correlate with ALS development later in life.

Although the correlation between BMAA ingestion and increased ALS incidence is the most well studied in Guam, it is by no means restricted to that area of the world. A 2009 study found residents of Enfield New Hampshire living around the adjoining lakes Crystal lake and Lake Mascoma, which exhibit frequent high levels of cyanobacterial blooms, have a 10–20× greater incidence of ALS than the general population (Caller *et al.*, 2009). In Qatar, cyanobacteria may make up to 56% of the desert crust that forms during dry seasons. A 2009 study of ALS incidence among U.S. veterans found those serving in the gulf during 1990–1991 had a significantly increased risk of developing ALS. Exposure to BMAA via cyanobacterial dust was likely heightened for Gulf War veterans by vehicular disturbance of the cyanobacterial crust (Cox *et al.*, 2009). A 2013 study showed that individuals living around Thao Lagoon, France had a significantly increased incidence of ALS that may be related to the high amounts of BMAA bioaccumulating in shellfish caught in the area and/or to aerosolization of BMAA off the lagoon due to high winds (Masseret *et al.*, 2013). A 2013 study of ALS patients in Annapolis Maryland linked their disease to the frequent consumption of blue crab, containing high levels of BMAA, caught in the Chesapeake Bay (Field *et al.*, 2013). Critically, a recent experimental study has shown that chronic dietary exposure to BMAA for 140d led to Neurofibrillary tangles and β-amyloid plaques in vervet monkeys (Cox *et al.*, 2016), indicating the potential for widespread neurological outcomes in exposed human populations.

There is presently no $LD_{50}$ or NOAEL for BMAA in either terrestrial or aquatic environments (Merel *et al.*, 2013), and the vast majority of BMAA exposure studies have only reported amount of BMAA per gram of plant or animal tissue. One report, however, has measured water-borne BMAA specifically in Nebraska freshwater public lakes and reservoirs with active HABs, and has found concentrations ranging from 1.8 to 24.5 μg/l (Al-Sammak *et al.*, 2014). Pathological late-onset neurodegeneration is increasingly being seen as beginning early in development, and therefore investigating phenotypic linkages between development and preclinical disease measures is vital to understanding eventual pathogenesis (Kovacs *et al.*, 2014).

Exposing an animal to a particular neurotoxin may result in acute and/or chronic neurological defects. Determining whether these are truly involved in a neurodegenerative disease like

ALS, however, is much harder to measure. We see the use of genetic animal models of ALS as a "sensitized" background on which to test the impact of toxin exposure on known timeframes of disease progression. Approximately 20% of FALS cases are due to mutations in the ubiquitously expressed Cu/Zn superoxide dismutase (SOD1) gene (Rosen *et al.*, 1993; Siddique *et al.*, 1991). To date, over 200 mutations within the 153 aa SOD1 protein have been found to be associated with FALS (https://ghr.nlm.nih.gov/gene/SOD1#conditions; last accessed January 30, 2017). Since the discovery of mutations in SOD1 in ALS patients, dozens of animal models with human ALS-linked mutations have been developed, including mice (Gurney *et al.*, 1994) and zebrafish (Ramesh *et al.*, 2010). A zebrafish ALS model has been developed carrying transgenes containing the endogenous zebrafish *sod1* promoter and *sod1* gene, with the 93rd amino acid mutated from glycine to arginine, a highly conserved amino acid often mutated in human ALS (Ramesh *et al.*, 2010). These fish exhibit many features of ALS, including larval and adult neuromuscular junction (NMJ) defects, decreased adult swimming endurance, and eventual age-dependent loss of motor neurons, paralysis, and death. We have therefore tested the impacts of environmentally-relevant doses of BMAA (0, 2.5, 5, 10, and 25 μg/l) on embryonic neurodevelopmental and early adult neurodegenerative phenotypes in these transgenic SOD1-ALS-zebrafish. Comparing phenotypic outcomes between genetic and genetic/environmental treatments can provide a more direct measure of how toxins may influence known neurological disease pathways, allowing us to distinguish between acute toxicities that are not involved in the disease course and those that are amplified due to the combination of genes and the environment. Here, we report genetic-environmental interactions in early neurodevelopmental alterations and on functional changes in adult-onset presymptomatic motor function in SOD1-G93R mutant ALS-zebrafish (Ramesh *et al.*, 2010) exposed embryonically to BMAA.

## MATERIALS AND METHODS

*Animals*. Adult zebrafish were maintained at the University of Maine zebrafish facility according to the Institutional Animal Care and Use Committee (IACUC) standards. Fertilized eggs for 30–72 hours postfertilization (hpf) experiments were collected at one cell stage before the start of experiments and raised in egg water (60 μg/l Instant Ocean Sea Salts) with 0.0003% methylene blue at 28.5°C. Transgenic embryos were subjected to 37°C heat shock for 2 h at 24 hpf for colocalization experiments or 6 hpf for nerve length experiments and sorted for their transgene by fluorescence.

Adult fish used in the long-term BMAA study were collected as embryos and exposed to 5 BMAA doses for the first 5 days of life. Fertilized eggs for adult experiments were collected at one cell stage before the start of experiments and raised in egg water (60 μg/l Instant Ocean Sea Salts) with 0.0003% methylene blue at 28.5 °C for 5 days. They were subjected to 37°C heat shock for 2 h at 24 hpf for transgenic sorting. At 5 days, they were placed in tanks at the University of Maine zebrafish facility and maintained according to IACUC standards until removed for spinning task experiments at 5 months.

*Transgenic animals*. Transgenic fish strains were donated by Dr Christine Beattie at Ohio State University. Their generation is described (Ramesh *et al.*, 2010). To sort for fluorescence, animals were heat shocked at 24 hpf (for 72 hpf and long-term experiments) or 6 hpf (for 30-h experiments). Heat shock was performed in a Techne thermocycler with 4 embryos per PCR tube. Heat shock consisted of a cycle of 30 min at 23°C followed by a cycle of 30 min at 37°C, repeated once. Embryos and larvae were screened for dsRed expression using a Zeiss screening microscope or Night Sea fluorescence adaptor on an Olympus SZ61.

*BMAA exposure*. A stock solution of 10 mg/ml BMAA (L-BMAA hydrochloride, Sigma) in DI water was diluted to 2.5, 5, 10, and 25 μg/l with egg water. Embryos were exposed after collection until euthanization. Egg water containing BMAA was changed daily.

*Staining of 30 hpf embryos*. Embryos were euthanized at 30 hpf with 300 mg/l Tricaine and fixed in 4% paraformaldehyde overnight at 4°C. Fixed embryos were then washed 6×, for 10 min with 1× PBS and blocked for 2 h at room temperature in 2% BSA, 0.5%, (increased to 1% in transgenic experiments) Triton-X 100 and 0.05% DMSO before incubation with the SV2 primary antibody (IgG1, 1:200; Developmental Studies Hybridoma Bank) in block overnight at 4°C. Embryos were then washed 6× for 10 min in 1× PBS and incubated with AlexaFluor-IgG1-488-conjugated antimouse secondary antibody (1:250; Life Technologies) for 2 h at room temperature, washed 6× for 10 min each using 1× PBS and mounted onto 25 × 75 × 1 mm glass slides from VWR Cat. No. 48311-600 with 24 × 40 mm cover glass from VWR Cat. No. 48393230 using Dako Fluorescent Mounting Media (Dako) and Pap Pen Hydrophobic Slide Marker (VWR).

*Staining of 72 hpf embryos*. Embyros treated for 72 hpf analysis had egg water changed every day. Larvae were euthanized at 72 hpf with 300 mg/l Tricaine and fixed in 4% paraformaldehyde overnight at 4°C. Fixed embryos were then permeabilized by successive incubations of DI water for 5 min, ice-cold acetone at −20°C for 60 min, DI water for 5 min. Larvae were washed 3× for 10 min with PBST (1–2% TritonX-100, 1× PBS) then incubated in PBDT buffer (1% DMSO, 1% BSA, 1–2% TritonX-100, 1× PBS), with 5% normal goat serum (in later experiments PBDT buffer was replaced with Block Buffer [2% BSA, 1% Triton-X 100, 0.05% DMSO]) for 60 min followed by incubation with Alexa 647-conjugated α-BTX (1:100; Molecular Probes) for 60 min at room temperature. Then larvae were washed 3× for 10 min with PBST. Larvae were incubated with primary mouse monoclonal anti-SV2 antibody (1:50) at 4°C overnight, then washed 6× for 10 min with PBST and incubated in Alexa-488 conjugated goat anti-mouse antibody (1:400) overnight at 4°C. Then larvae were washed 4×, 10 min in PBST, then incubated in DAPI (1:100) for 10 min. Embryos were washed 1× with PBST for 10 min then mounted. In later experiments, staining quality was increased by agitating larvae during washes using VWR Multimix 13916-822, and by increasing SV2 and Alexa-488 goat anti-mouse antibody incubation period to ≤7 days.

*Imaging and image analysis*. Fluorescent images of embryos were taken using confocal microscopy on an Olympus FV-1000 with a 20× objective. All embryos and larvae were flat mounted using Dako Fluorescent Mounting Media (Dako). 30 hpf nerve length analysis was performed using the FIJI simple neurite tracer on the 10 nerves on each side of the body wall beginning at the end of the yolk sac extension and continuing towards the superior end (Supplementary Figure 1). NMJ colocalization (Supplementary Figure 2) in 72 hpf fish was analyzed using FIJI software. Images with aberrant background staining of the

neural tube were cropped and a 5-chevron portion of each larva was used for analysis. The Pearson's Correlation Coefficient was calculated by FIJI between presynaptic SV2 Alexa-488 and postsynaptic α-BTX Alexa-647 staining.

*Spinning task*. To assess 5-month-old adult zebrafish endurance we followed a previously established protocol for assessing adult zebrafish motoneuron function (Blazina *et al.*, 2013). We amended the protocol to account for the relative smaller size and weaker swimming ability of our 5-month-old fish compared with the ≥8-month fish assessed by Blazina *et al.* (2013). We lowered the rpm of the stir bar in our tests to accommodate the slower swimming speed of our younger fish. Fish were individually tested in a 1 L beaker containing 800 ml of Umaine zebrafish facility water heated to 27.5°C. The beaker was surrounded by cardboard to avoid external visual interference and increased fear, and zebrafish were allowed to acclimate to the beaker for at least 2 min before testing began. Three tests were performed in which the zebrafish was monitored and timed as it swam against a current generated by a 9 × 50 mm stir bar at 120 rpm. The stir bar and timer were stopped at the point when the fish was no longer able to swim against the current and lost its swimming orientation in the beaker.

*Statistics*. All statistics for nerve length and colocalization analysis were performed using analysis of variance (ANOVA) (JMP, Version 11. SAS Institute Inc., Cary, North Carolina, 1989-2007). Linear regressions for swimming endurance and fatigability were performed using JMP. FIJI simple neurite tracer length was taken for 10 nerves per fish per side. Two-way ANOVA and Dunnetts multiple comparison tests were used to determine differences between control (0) and all treatments. Differences between treatment groups were considered significant if $P < .05$.

## RESULTS

### *BMAA Dose-Wise Experiments across Genotypes and Developmental Stages*

Experiments were performed on a zebrafish ALS transgenic model and paired wild type (WT) transgenic control. The transgenic models, TG-z*sod1* WT and TG-z*sod1* G93R (Ramesh *et al.*, 2010), overexpress zebrafish WT Sod1 and mutant ALS-linked Sod1-G93R, respectively. We used these models to examine 2 early ALS-linked neurodevelopmental phenotypes in the zebrafish embryos and larvae: 30 hpf motor nerve length and 72 hpf NMJ colocalization. We also examined adult ALS behavioral phenotypes (swimming endurance and fatigability) in the 5-month adult zebrafish. As nontransgenic controls, we examined embryonic and larval phenotypes in AB control zebrafish, and adult phenotypes in the nontransgenic siblings of our 5-month transgenic zebrafish. Due to limitations in our transgenic fish facilities we were not able to age to adulthood fish exposed to 10 or 25 μg/l BMAA.

### *Altered 30 hpf Nerve Development as an Early Phenotypic Effect*

Previous research (Ramesh *et al.*, 2010) has detected the presence of an early neurological phenotype (11 days postfertilization [dpf] reduction of NMJ pre- and postsynaptic colocalization) for transgenic TG-z*sod1* G93R compared with TG-z*sod1* WT zebrafish. Zebrafish embryos have significantly shortened nerves at 30 hpf when transiently overexpressing ALS-causing mutant human proteins SOD1 G93A (Lemmens *et al.*, 2007), A4V (Lemmens *et al.*, 2007), and G37R (Lemmens *et al.*, 2007), TDP-43 G348C (Armstrong and Drapeau, 2013; Kabashi *et al.*, 2010; Vaccaro *et al.*, 2012), A315T (Kabashi *et al.*, 2010), and A382T (Kabashi *et al.*, 2010), and FUS R521H (Vaccaro *et al.*, 2012). This has not, however, been reported in the transgenic TG-z*sod1* G93R model. We sought to determine whether BMAA exposure could trigger detectable changes in nerve length at 30 hpf in the transgenic embryos. We treated TG-z*sod1* G93R embryos, AB embryos, and TG-z*sod1* WT embryos with 5 doses of BMAA (0, 2.5, 5.0, 10.0, and 25.0 μg/l) from the 1-cell fertilized egg stage until they were euthanized at 30 hpf, and measured motor neuron length (Supplementary Figure 1) within each genotype across the spectrum of BMAA doses and between genotypes at each BMAA dose.

For AB embryos, there was a significant reduction in mean nerve length compared with controls at all doses of BMAA above 5 μg/l (Figure 1A). Nerve length for TG-z*sod1* G93R fish was significantly lengthened with exposure to 2.5 μg/l and significantly shortened at 10 μg/l BMAA (Figure 1B), while nerve length in the TG-z*sod1* WT fish was unaffected by any BMAA exposure (Figure 1C). This indicates that the genetic insult of TG-z*sod1* G93R overexpression results in a bi-modal threshold for nerve alterations. Both TG-z*sod1* WT and TG-z*sod1* G93R embryos had significantly shorter motor neurons than AB controls (data not shown), likely due to differing genetic backgrounds between the AB and the Tg fish lines. We found that Tg-WT fish have significantly longer nerves than Tg-G93R at all BMAA doses except at 2.5 μg/l (Figure 1D), where the G93R fish showed a significantly increased nerve length (Figure 1B). Overall, we have established 30 hpf mean nerve length in TG-z*sod1* G93R as a new early phenotype effected by BMAA exposure in this transgenic zebrafish model of ALS.

### *Altered NMJ Colocalization as an Early Phenotypic Effect*

Previous research has established that a reduction in colocalization at the 11 dpf NMJ was correlated with late stage ALS-like phenotypes including loss of endurance, motor neuron loss, sporadic paralysis, and early death (Ramesh *et al.*, 2010). NMJ colocalization values are scored using Pearson's correlation coefficient, a statistic measuring correlation of localization of pixels between 2 fluorescent channels (perfect colocalization = 1, perfect unrelatedness = 0). We examined NMJ colocalization (Supplementary Figure 2) at 72hpf to determine whether an earlier time-point could be affected by BMAA exposure. For AB fish, all doses of BMAA resulted in higher NMJ colocalization values than controls (Figure 2A). For TG-z*sod1* G93R embryos (Figure 2B), there is a significant increase in NMJ colocalization at 10 and 25 μg/l BMAA compared with the controls (0 μg/l BMAA). This dose-dependent pattern of changes in 72hpf colocalization was distinct from the dose-dependent pattern of BMAA-induced changes in 30 hpf nerve length in the TG-z*sod1* G93R (Figure 1B), where a low dose (2.5 μg/l) resulted in longer nerves and a higher dose (10 μg/l) resulted in decreased nerve length. For TG-z*sod1* WT, (Figure 2C) BMAA has no effect on NMJ colocalization, consistent with our finding that BMAA exposure had no effect on 30hpf nerve length (Figure 1C). There were significant differences in NMJ colocalization between G93R and WT genotypes at the higher doses of 10 ($P = .0010$) and 25 μg/l ($P = .0126$) BMAA (Figure 2D), with G93R having higher Pearson's values at both doses.

### *BMAA Exposure and Establishing Loss of Endurance Symptoms in the 5-Month-Old Zebrafish*

Ramesh *et al.* (2010) reported that the TG-z*sod1* G93R fish develops ALS-like motor symptoms in adulthood. These symptoms

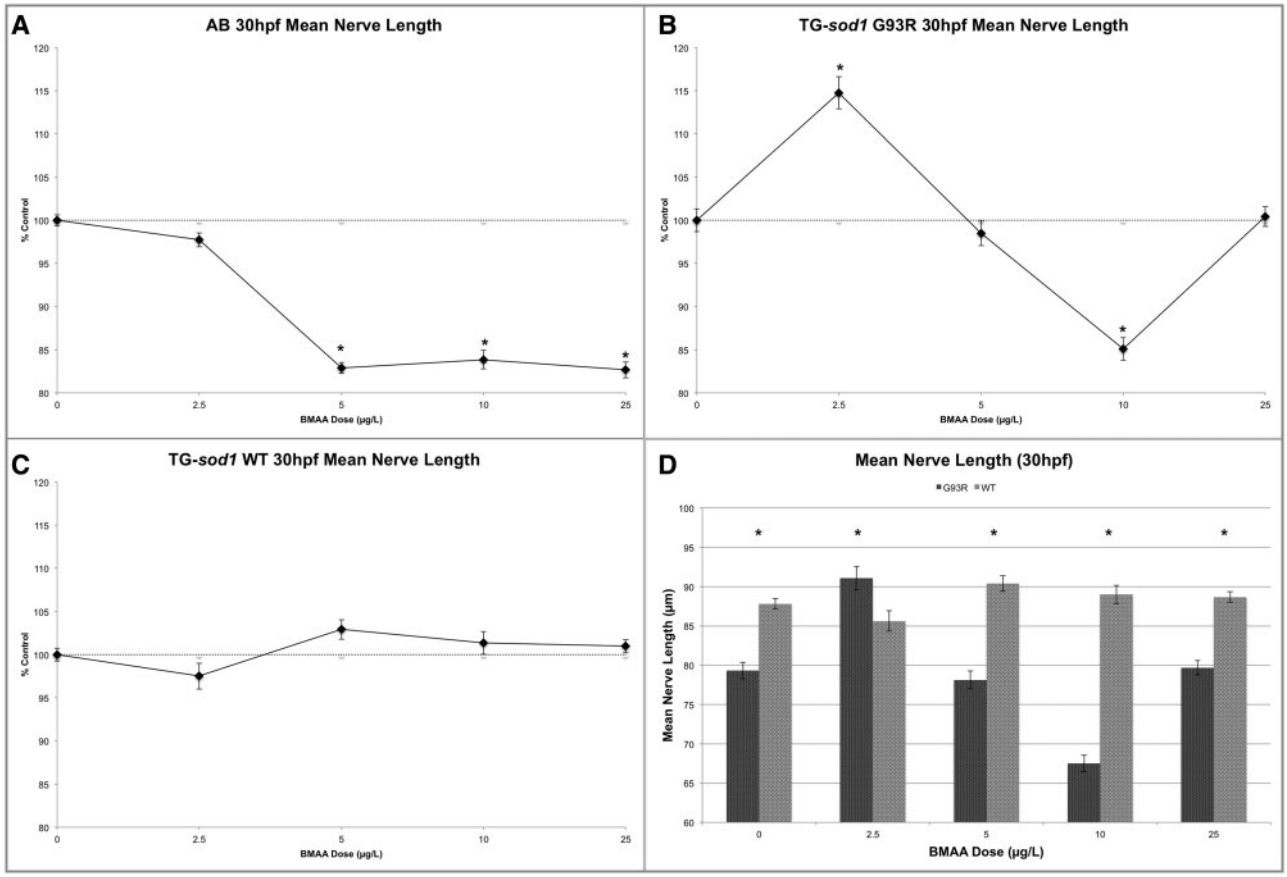


FIG. 1. Mean nerve length in 30hpf transgenic embryos is significantly altered between treatments and genotypes. Asterix (*) indicates significantly different than control (0 µg/l BMAA) according to Dunnetts Multiple Comparison. **A,** Nerve length within AB embryos is significantly shortened at high BMAA doses (5–25 µg/l) compared with controls (each $P < .001$). **B,** Nerve length within TG-z*sod1* G93R is significantly increased at 2.5 µg/l and significantly shortened at 10 µg/l BMAA compared with controls (each $P < .001$). **C,** Nerve length within TG-z*sod1* WT embryos is unaffected by the BMAA doses tested (all $P > .11$). **D,** There are significant differences in overall nerve length between G93R and WT genotypes at each dose. Tg-G93R embryos have significantly shorter nerves at all doses except at 2.5 µg/l, where they are significantly longer than Tg-WT. Asterix (*) indicates significant difference between G93R and WT within each dose ($P < .001$ for all comparisons).

include loss of swimming endurance (12 months) and paralysis, motor neuron loss, and early death (>16 months). We explored how early embryonic BMAA exposure could affect endurance in the adult zebrafish at presymptomatic stages. TG-z*sod1* G93R and TG-z*sod1* WT embryos (and their nontransgenic siblings, referred to here as NTG-z*sod1* G93R and NTG-z*sod1* WT, respectively) were exposed to 0, 2.5, or 5 µg/l BMAA for the first 5 days of life and were then raised under standard conditions in the University of Maine zebrafish facility. At 5 months of age, we quantified their endurance and fatigability using a modified version of the spinning task protocol (Blazina *et al.*, 2013).

To quantify endurance, 10 fish were selected from each treatment group and timed in 3 consecutive trials as they swam against a current generated in a 1000 ml beaker containing 800 ml water with a stir bar spinning at 120 rpm. Each trial was deemed over when the fish lost its orientation while attempting to swim against the current. There is a significant reduction in endurance in the 5-month-old TG-z*sod1* G93R fish exposed to the highest dose (5 µg/l BMAA) (Figure 3A) and a significant increase in endurance in TG-z*sod1* WT (Figure 3B) fish compared with controls. At 0 and 5 µg/l BMAA exposure, there are no significant differences in swimming ability between TG-z*sod1* WT and TG-z*sod1* G93R 5 month fish, while WT fish swim significantly better than G93R fish after having been exposed to 5 µg/l BMAA during the first week of life (Figure 3C). Linear regression shows that there are significant trends in which increasing BMAA exposure causes an increase in swimming ability among TG-z*sod1* WT fish and at the same time causes a significant decrease in swimming ability among TG-z*sod1* G93R fish (Figure 3D). This indicates that although there is no detectable difference in swimming endurance between the transgenic fish based on genotype alone (in the absence of BMAA exposure) at this presymptomatic stage, the interaction between BMAA and genotype is able to drive a significant difference in this phenotype with increasing dose.

Swimming endurance was also examined for the nontransgenic siblings at each BMAA dose tested and we found that NTG-z*sod1* WT had improved swimming when exposed to 2.5 or 5 µg/l BMAA compared with controls, while NTG-z*sod1* G93R showed no difference with BMAA exposure compared with controls (Figure 3E). This may be due to the fact that the WT strain had poorer dsRED expression and was more difficult to sort during embryo development, and therefore some of the TG animals may have mistakenly been labeled nonTG. The lack of any change in the NTG-z*sod1*-G93R siblings indicates that the changes in endurance in the TG-z*sod1*-G93R animals are not driven solely by the application of BMAA, but rather is from the negative interaction of SOD1-G93R and BMAA.

### *Establishing Fatigability Symptoms in the 5-Month-Old Zebrafish*

Increased fatigability is seen in human ALS patients, with one report showing that over the course of 25 min of exercise, voluntary and tetanic force declined more sharply in ALS

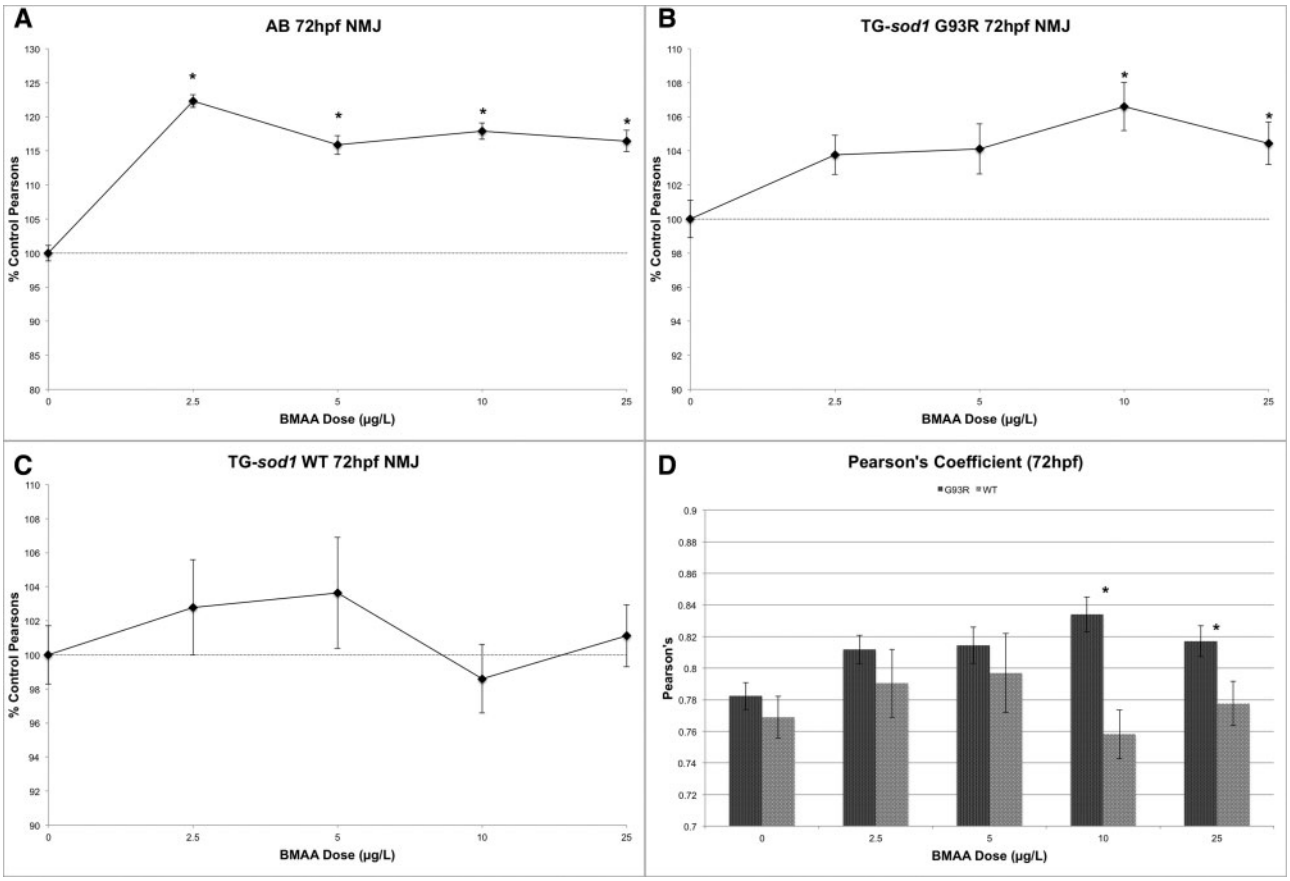


FIG. 2. NMJ colocalization in 72 hpf transgenic larvae is significantly between treatments and genotypes. Asterix (*) indicates significantly different than control (0 µg/l BMAA) according to Dunnetts Multiple Comparison. **A,** NMJ colocalization in AB embryos is significantly higher with all BMAA doses compared with control (all $P < 0.0001$). **B,** NMJ colocalization within TG-zsod1 G93R larvae significantly increases at 10 µg/l ($P = .0027$) and 25 µg/l BMAA ($P = .0185$) BMAA. **C,** NMJ colocalization within TG-zsod1 WT larvae is unaffected by BMAA dose ($P = .5587$). **D,** NMJ colocalization differs significantly between genotypes depending on BMAA dose. TG-zsod1 G93R embryos have significantly higher colocalization than TG-zsod1 WT larvae at 10 µg/l ($P = .0010$) and 25 µg/l ($P = .0261$) BMAA.

patients than in controls, indicating increased fatigue (Sharma *et al.*, 1995). Fatigability has not previously been reported as a potential ALS-like phenotype in the zebrafish, however Ramesh *et al.* did report that Tg-G93R fish that failed in the swim test did not recover well (Ramesh *et al.*, 2010). To determine fatigability, we examined how performance changed across 3 consecutive swimming trials for each fish. We found that TG-z*sod1* G93R zebrafish (Figure 4A) show significant fatigue after early exposure to 0 or 5 µg/l BMAA while TG-z*sod1* WT fish (Figure 4B) show no significant fatigue regardless of early BMAA exposure. This recapitulates the trend seen in TG-z*sod1* G93R 30hpf mean nerve length. This may also indicate that 30 hpf mean nerve length in the TG-z*sod1* G93A and TG-z*sod1* WT zebrafish is a predictor of these adult ALS-like phenotypes. Because this phenotype is present only in the TG-z*sod1* G93R model, we believe this is a phenotype that can be used to monitor ALS-like symptoms in the zebrafish at the preclinical stage in future toxicological studies.

### *Overexpression of WT SOD1 Provides Neuroprotection*

If overexpression of Sod1WT was independent of any BMAA induced phenotypic changes, we would expect to see identical results in the AB and TG-zsod1 WT models. NMJ colocalization and nerve growth in the embryonic/larval AB zebrafish was altered by exposure to BMAA. However, these BMAA dose-dependent changes in phenotype were not recapitulated in the TG-zsod1 WT model. This apparent neuroprotective function of WT SOD1, may also be seen when comparing the 5-month swim endurance between TG-z*sod1* WT zebrafish and their nontransgenic siblings. Endurance in the 5-month TG-z*sod1* WT zebrafish was actually improved after early 5 µg/l BMAA dose. This increase in endurance is only seen in TG-z*sod1* WT zebrafish. There is no change in endurance over doses tested in the nontransgenic siblings TG-z*sod1* G93R, and TG-z*sod1* G93R endurance decreases with BMAA dose. This overcompensation for early BMAA exposure may be mediated by SOD1 WT overexpression.

## DISCUSSION

Developmental plasticity, whereby one genotype gives rise to multiple phenotypic outcomes in response to environmental and/or genetic disruptions, is proposed to be the mechanism by which early developmental defects can lead to late-onset disease states or lead to altered disease course over time (Barker, 2004; Coppede *et al.*, 2006; Sasaki and Kishi, 2013; Vaiserman, 2011). Early defects in neural circuitry have been found to be associated with late-onset neurological disorders, including both cognitive and degenerative diseases (Fox *et al.*, 2012; Kovacs *et al.*, 2014; Tang *et al.*, 2014). Recently, key genes required for human neuronal differentiation during fetal development were also identified as genes involved in late-onset neurodegeneration, highlighting the mechanistic link between

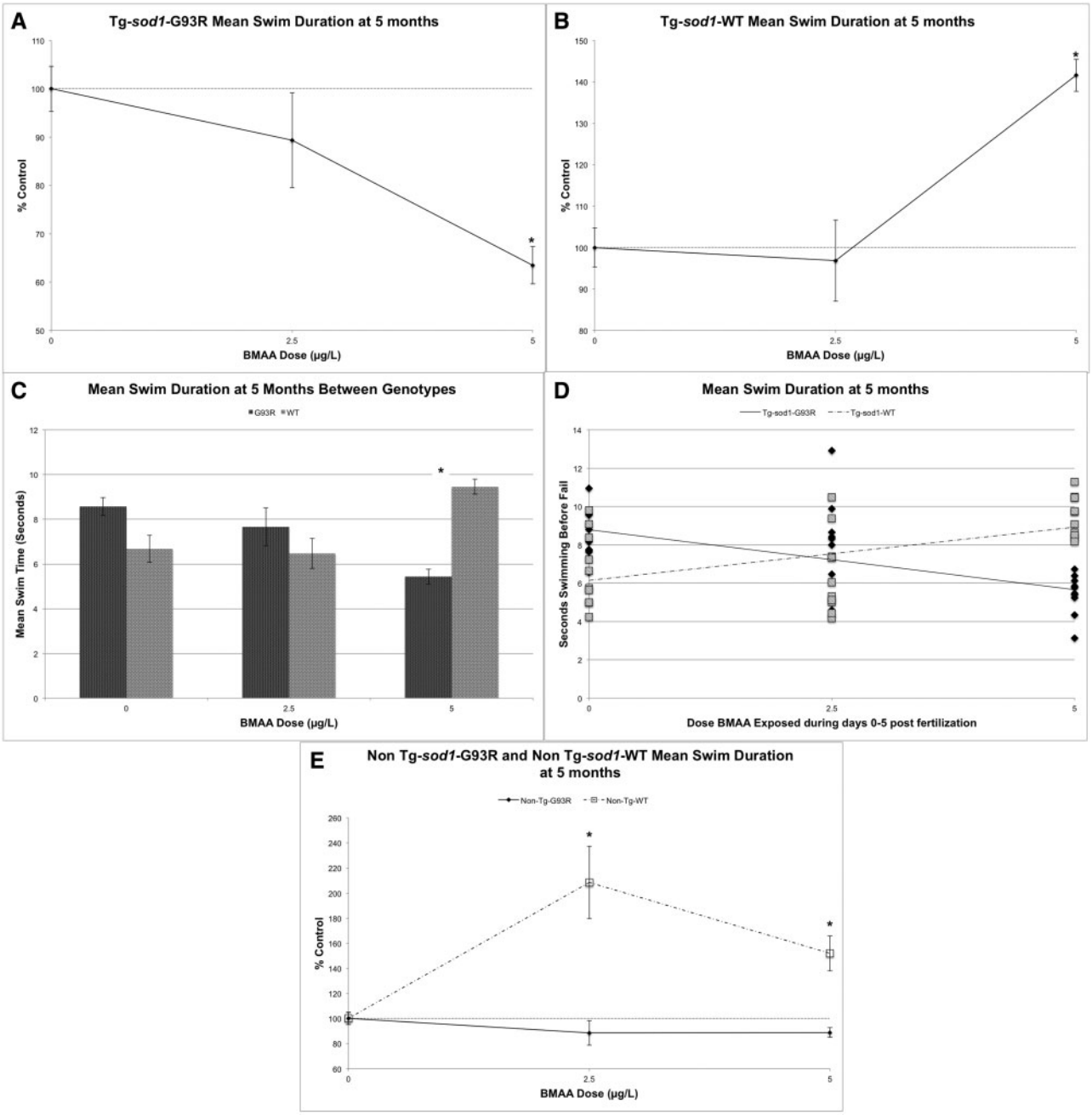


**FIG. 3.** Swimming endurance at 5 months is significantly altered by BMAA dose within and between genotypes. **A,** Endurance within TG-z*sod1* G93R fish is significantly reduced after early exposure to 5 µg/l BMAA ($P = .019$). **B,** Endurance within TG-z*sod1* WT fish is significantly increased after early BMAA exposure to 5 µg/l ($P = .0010$). **C,** Endurance between TG-z*sod1* G93R and TG-z*sod1* WT is significantly altered after early exposure to 5 µg/l BMAA, with TG-z*sod1* WT having significantly better endurance (* indicates significant difference between genotypes at each treatment dose). **D,** Linear regression indicates a significant decrease ($P = .0006$, $y = -0.8267x + 8.7946$, $R^2 = 0.351$) in endurance in TG-z*sod1* G93R with BMAA dose and a significant increase ($P = .0028$, $y = 5556x + 6.1462$, $R^2 = 0.277$) in endurance in TG-z*sod1* WT with BMAA dose. **E,** There is no significant difference between NTG-z*sod1* G93R sibs exposed to BMAA and controls, while NTG-zsod1-WT sibs show increased swimming at 2.5 and 5 µg/l BMAA exposure compared with controls (* indicate significant difference from 0 BMAA control within each genotype).

pathways necessary for early neuronal development and late-onset disease (Iglesias *et al.*, 2013; Zhang *et al.*, 2013), and of the utility of examining early embryonic defects as surrogates and precursors for adult clinical-stage neurodegenerative diseases. Potentially, mutations in genes crucial to early neuronal differentiation make neurons more susceptible to environmental disruptions, leading to late-onset disease (Modgil *et al.*, 2014; Zhang *et al.*, 2013). Early toxicological environmental exposures can also result in epigenetic changes that impact gene expression patterns later in life, leading to a later-onset disease state (Reamon-Buettner *et al.*, 2008).

One environmental neurotoxin linked through epidemiological studies to ALS is BMAA. BMAA exposure through ingestion (Banack and Murch., 2009) or inhalation (Cox *et al.*, 2009) has been associated with an increased prevalence of ALS in exposed human populations. BMAA is a nonproteinogenic amino acid. It is an analog of glutamate, capable of binding postsynaptic NMDA and AMPA/kainite glutamate receptors (Vyas and Weiss,

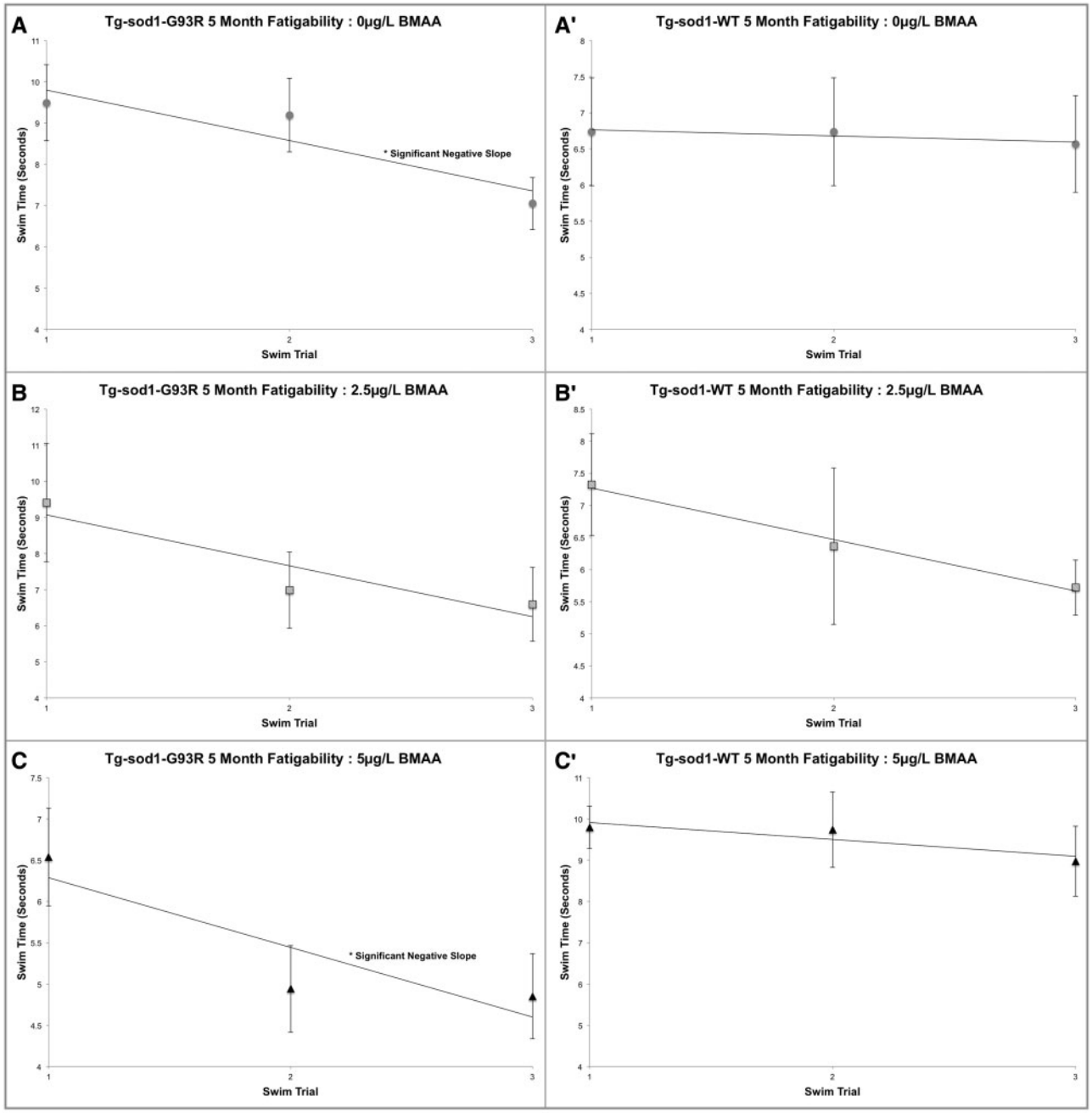


**FIG. 4.** Fatigability in 5-month-old zebrafish differs increases in Tg-zsod1-G93R but not in Tg-zsod1-WT fish. **A–C**, Linear regression reveals that there is are significant increases in fatigability as the no. of swim challenges (trials) increase in TG-z*sod1* G93R zebrafish exposed embryonically to 0 and 5 µg/l BMAA. **A′–C′**, Linear regression reveals that TG-z*sod1* WT zebrafish do not become significantly fatigued regardless of BMAA dose tested. (A) $P = .0442^*$, $y = -1.222x + 11.02$, $R^2 = 0.137$. (B) $P = .1241$, $y = -1.411x + 10.48$, $R^2 = 0.082$. (C) $P = .0381^*$, $y = -0.844x + 7.13$, $R^2 = 0.145$. (A′) $P = .8664$, $y = -0.085x + 6.85$, $R^2 = 0.001$. (B′) $P = .1983$, $y = -0.802x + 8.07$, $R^2 = 0.058$. (C′) $P = .4495$, $y = -0.404x + 10.31$, $R^2 = 0.022$.

2009). It is known to be produced by cyanobacteria, diatoms (Jiang *et al.*, 2014), and dinoflaggelates (Lage *et al.*, 2014). It is prolifically produced worldwide in freshwater (Al-Sammak *et al.*, 2014; Esterhuizen and Downing, 2008; Metcalf *et al.*, 2008), estuarine (Cervantes Cianca *et al.*, 2012), and saltwater environments (Brand *et al.*, 2010). BMAA can exist freely as an amino acid or become incorporated into protein (Karamyan and Speth, 2008; Metcalf *et al.*, 2008) and has been found in plant and animal tissue at various trophic levels. *In vitro*, BMAA has been shown to incorporate into human proteins in place of L-serine (Dunlop *et al.*, 2013). The incorporation of nonproteinogenic amino acids into protein is not unique to BMAA, it has also been shown to occur with other nonproteinogenic amino acids: canavanine and L-Dopa (Rodgers and Shiozawa, 2008). This phenomenon occurs when nonproteinogenic amino acids are mistakenly bound to an amino tRNA synthetase in place of an animo acid structural analog (Dunlop *et al.*, 2013).

Since they were first reported in 1883, toxin producing cyanobacterial blooms have been increasing in incidence, duration, and intensity largely because of declining water quality worldwide (Carmichael, 2008). Therefore, the impact of climate and eutrophication, which contribute directly to increased incidence

of bloom formation, on cyanobacterial blooms is of critical importance to the exposure of human populations to BMAA. Increasing nutrient enrichment (eutrophication) of water bodies associated with human industrial and agricultural methods favors (Paerl and Paul, 2012) HABs and is correlated with increased cyanobacterial growth (Vahtera *et al.*, 2007). Because eutrophication is primarily caused by human activities, incidence of toxic cyanobacterial blooms is essentially the same worldwide, with some increases in toxic blooms in areas of warmer climates. In addition to eutrophication, climate change (global warming) is suspected to favor cyanobacterial growth by leading to increases in metabolism, growth rates, and bloom formation (Paerl and Paul, 2012). According to the International Panel on Climate Change (IPCC, 2014), global temperatures are expected to rise by as much as 2.5–7.8 °C by 2100. The prokaryotic cyanobacteria flourish at higher temperatures than their eukaryotic planktonic algae competitors. Therefore, a global increase in temperature may select for cyanobacterial growth and proliferation (Butterwick *et al.*, 2004).

*Drosophila* larvae fed BMAA show delayed and progressive neurotoxicity in adults (Zhou *et al.*, 2010), and BMAA has high placental transfer into the brain of embryonic rodents through exposure to pregnant females (Karlsson *et al.*, 2009a), with neonatal exposure resulting in permanent changes in postnatal neural development and long-term adult cognitive impairments (Dawson *et al.*, 1998; Karlsson *et al.*, 2009b, 2011, 2013). BMAA is excreted in milk during lactation (Andersson *et al.*, 2013), a key route of exposure for neonates during a particularly sensitive timepoint (Karlsson *et al.*, 2009a,b, 2011, 2012). These examples indicate that early human exposure to BMAA may have drastic consequences on susceptible individuals. We find that early exposure to BMAA results in embryonic nerve development defects in a dose-dependent manner in mutant overexpressing G93R-SOD1 fish, and that these early defects are reflected in adult presymptomatic swimming capability. Interestingly, we find that there is no impact on these measures in fish overexpressing the unmutated WT SOD1, and that in some measures the WT-SOD1 fish actually improve, implying a possible protective role against neurodegeneration. Our results show that early exposure to BMAA has genotype-specific effects on early neurodevelopment and on adult motor function.

Our studies of gene–environment interactions have identified new influences on the development of ALS-like phenotypes, and have expanded on our understanding of previously established early indicators of ALS and human-like presymptomatic stage ALS symptoms in the zebrafish. These phenotypes are extremely heterogeneous, similar to the wide range of phenotypes seen in SALS and FALS patients (Kiernan *et al.*, 2011). Interestingly, these embryonic alterations are not linearly dose-dependent, but rather show more complex bell-shaped/biphasic or multi-phasic response. This is not surprising, as the combination of genetics and toxins could potentially result in multiple molecular and biochemical interactions. In fact, developmental exposure of zebrafish to methymercury has been shown to result in biphasic behavioral alterations, with mid-range doses having greater effects than low or higher doses (Weber, 2006). Similarly, embryonic exposure to lead (Pb) results in multiphasic dose by age-specific GABAergic gene expression and GABA protein level responses (Wirbisky *et al.*, 2014). These bi- and multi-phasic responses to biological plasticity, also described as a hormetic response, have been extensively described in neurodevelopmental and neurological disease experimentation (Calabrese, 2008; Calabrese *et al.*, 2010; Calabrese and Mattson, 2011). These responses overall are generalizable and independent of the biological model, the endpoint being measured, or the chemical class being used, and have been observed for chemicals, metals, herbicides and radiation exposures(Calabrese, 2013). These responses are reflected in our 30 and 72 hpf phenotypic measures, with medium doses having a larger impact than low or high doses.

These results were interesting to us because although one might expect a simple linear effect of dose, the bell shape of the responses indicates that there are indeed gene-toxin specific interactions that have previously been unreported. These sorts of nonlinear toxicological effects can be seen with other multiple-insult acute toxicity studies, but to our understanding, this is the first study looking at the impact over long-term development of an adult-onset disease. This study highlights the importance of investigating low-dose exposures in the context of genetic mutations, as the potential for compensation interactions is important. Our results in this initial study will allow us to look for biochemical/molecular pathways at the impacted doses that might point to mechanisms of function.

Toxicity and drug studies in the zebrafish are cost effective, fast, and easily regulated, particularly during early development, making drug and toxicity studies in zebrafish genetic disease models ideal (Chakravarthy *et al.*, 2014). By establishing the correlation of earlier biomarkers for ALS with late stage ALS human-like phenotypes in the zebrafish, we can improve the potential for drug and toxicity screening. It has been proposed that measures of eventual functional impairment can be a more complex response to early toxin exposure than acute mortality or structural alterations (Samson *et al.*, 2001). We have identified phenotypic changes at earlier developmental time points than have previously been reported for both NMJ alterations and for adult swimming endurance, and we have established 30 hpf mean nerve length reduction and increased fatigability in the 5-month-old zebrafish as new disease phenotypes in the TG-z*sod1* G93R and TG-z*sod1* WT models. TG-z*sod1* WT overexpression results in stable phenotypes after BMAA exposure when compared with AB phenotypes, which show dose-dependent phenotypic changes. Critically, we have found that early exposure to BMAA results in a decrease in swimming endurance and an increase in fatigability in 5-month-old TG-z*sod1* G93R fish, and that these behavioral adult defects are reflected early on in embryonic development. This study contributes to our understanding of neurodegeneration as a multi-hit, long-term progression of disease that may begin with alterations in neural circuitry early in life (Tartaglione *et al.*, 2016).

## SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

## ACKNOWLEDGMENTS

We thank Mark Nilan for maintenance of zebrafish colonies. We thank Dr Clarissa Henry and Dr Julie Gosse for critical reading of the article. We thank Michelle Goodey for technical assistance.

## FUNDING

Funding was supplied by the University of Maine (startup funds for R.S.) and from a gift from the WaterWheel Foundation.

## REFERENCES


Al-Chalabi, A., Calvo, A., Chio, A., Colville, S., Ellis, C. M., Hardiman, O., Heverin, M., Howard, R. S., Huisman, M. H. B., Keren, N., *et al.* (2014). Analysis of amyotrophic lateral sclerosis as a multistep process: A population-based modelling study. *Lancet Neurol.* **13**, 1108–1113.

Al-Sammak, M. A., Hoagland, K. D., Cassada, D., and Snow, D. D. (2014). Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants. *Toxins* **6**, 488–508.

Andersen, P. M. (2006). Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr. Neurol. Neurosci. Rep.* **6**, 37–46.

Andersen, P. M., and Al-Chalabi, A. (2011). Clinical genetics of amyotrophic lateral sclerosis: What do we really know? *Nat. Rev. Neurol.* **7**, 603–615.

Andersson, M., Karlsson, O., Bergstrom, U., Brittebo, E. B., and Brandt, I. (2013). Maternal transfer of the cyanobacterial neurotoxin beta-N-methylamino-L-alanine (BMAA) via milk to suckling offspring. *PLoS One* **8**, e78133.

Armstrong, G. A., and Drapeau, P. (2013). Calcium channel agonists protect against neuromuscular dysfunction in a genetic model of TDP-43 mutation in ALS. *J. Neurosci.* **33**, 1741–1752.

Banack, S. A., and Cox, P. A. (2003). Biomagnification of cycad neurotoxins in flying foxes. *Neurology* **61**, 387–389.

Banack, S. A., and Murch, S. J. (2009). Multiple neurotoxic items in the Chamorro diet link BMAA with ALS/PDC. *Amyotroph. Lateral Scler.* **10(Suppl 2)**, 34–40.

Barker, D. J. P. (2004). Developmental origins of adult disease. *J. Am. College Nutr.* **23**, 588S–595S.

Bensimon, G., Lacomblez, L., and Meininger, V. (1994). A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N. Engl. J. Med.* **330**, 585–591.

Blazina, A. R., Vianna, M. R., and Lara, D. R. (2013). The spinning task: A new protocol to easily assess motor coordination and resistance in zebrafish. *Zebrafish* **10**, 480–485.

Bradley, W. G., Borenstein, A. R., Nelson, L. M., Codd, G. A., Rosen, B. H., Stommel, E. W., and Cox, P. A. (2013). Is exposure to cyanobacteria an environmental risk factor for amyotrophic lateral sclerosis and other neurodegenerative diseases? *Amyotroph. Lateral Scler. Frontotemporal Degener.* **14**, 325–333.

Brand, L. E., Pablo, J., Compton, A., Hammerschlag, N., and Mash, D. C. (2010). Cyanobacterial Blooms and the Occurrence of the neurotoxin beta-N-methylamino-L-alanine (BMAA) in South Florida Aquatic Food Webs. *Harmful Algae* **9**, 620–635.

Brooks, B. R. (1994). El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. Subcommittee on Motor Neuron Diseases/Amyotrophic Lateral Sclerosis of the World Federation of Neurology Research Group on Neuromuscular Diseases and the El Escorial "Clinical limits of amyotrophic lateral sclerosis" workshop contributors. *J. Neurol. Sci.* **124(Suppl)**, 96–107.

Bruijn, L. I., Miller, T. M., and Cleveland, D. W. (2004). Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu Rev Neurosci* **27**, 723–749.

Butterwick, C., Heaney, S. I., and Talling, J. F. (2004). Diversity in the influence of temperature on the growth rates of freshwater algae, and its ecological relevance. *Freshwater Biol.* **50**, 291–300.

Calabrese, E. J. (2008). Neuroscience and hormesis: Overview and general findings. *Crit. Rev. Toxicol.* **38**, 249–252.

Calabrese, E. J. (2013). Hormetic mechanisms. *Crit. Rev. Toxicol.* **43**, 580–606.

Calabrese, E. J., and Mattson, M. P. (2011). Hormesis provides a generalized quantitative estimate of biological plasticity. *J. Cell Commun. Signal.* **5**, 25–38. 10.1007/s12079-011-0119-1.

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Calabrese, E. J., and Mattson, M. P. (2010). Cellular stress responses, the hormesis paradigm, and vitagenes: Novel targets for therapeutic intervention in neurodegenerative disorders. *Antioxid. Redox. Signal.* **13**, 1763–1811.

Caller, T. A., Doolin, J. W., Haney, J. F., Murby, A. J., West, K. G., Farrar, H. E., Ball, A., Harris, B. T., and Stommel, E. W. (2009). A cluster of amyotrophic lateral sclerosis in New Hampshire: A possible role for toxic cyanobacteria blooms. *Amyotroph. Lateral Scler.* **10**, 101–108.

Caller, T. A., Field, N. C., Chipman, J. W., Shi, X., Harris, B. T., and Stommel, E. W. (2012). Spatial clustering of amyotrophic lateral sclerosis and the potential role of BMAA. *Amyotroph. Lateral Scler.* **13**, 25–32.

Carmichael, W. (2008). A world overview — One-hundred-twenty-seven years of research on toxic cyanobacteria — Where do we go from here? In *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs* (H. K. Hudnell, Eds.), pp. 105–125. Springer, New York, NY.

Cervantes Cianca, R. C., Baptista, M. S., Lopes, V. R., and Vasconcelos, V. M. (2012). The non-protein amino acid beta-N-methylamino-L-alanine in Portuguese cyanobacterial isolates. *Amino Acids* **42**, 2473–2479.

Chakravarthy, S., Sadagopan, S., Nair, A., and Sukumaran, S. K. (2014). Zebrafish as an in vivo high-throughput model for genotoxicity. *Zebrafish* **11**, 154–166.

Coppede, F., Mancuso, M., Siciliano, G., Migliore, L., and Murri, L. (2006). Genes and the environment in neurodegeneration. *Biosci. Rep.* **26**, 341–367.

Cox, P. A., Davis, D. A., Mash, D. C., Metcalf, J. S., and Banack, S. A. (2016). Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain. *Proc. R. Soc. B.* **283**, 20152397.

Cox, P. A., Richer, R., Metcalf, J. S., Banack, S. A., Codd, G. A., and Bradley, W. G. (2009). Cyanobacteria and BMAA exposure from desert dust: A possible link to sporadic ALS among Gulf War veterans. *Amyotroph. Lateral Scler* **10(Suppl 2)**, 109–117.

Dawson, R., Marschall, E. G., Chan, K. C., Millard, W. J., Eppler, B., and Patterson, T. A. (1998). Neurochemical and neurobehavioral effects of neonatal administration of B-Methylamino-L-Alanine and 3,3'-iminodipropionitrile. *Neurotoxicol. Teratol.* **20**, 181–192.

Dunlop, R. A., Cox, P. A., Banack, S. A., and Rodgers, K. J. (2013). The non-protein amino acid BMAA is misincorporated into human proteins in place of l-serine causing protein misfolding and aggregation. *PLoS One* **8**, e75376–e75310.

Erdner, D. L., Dyble, J., Parsons, M. L., Stevens, R. C., Hubbard, K. A., Wrabel, M. L., Moore, S. K., Lefebvre, K. A., Anderson, D. M., Bienfang, P., *et al.* (2008). Centers for Oceans and Human Health: A unified approach to the challenge of harmful algal blooms. *Environ. Health* **7(Suppl 2)**, S2.

Esterhuizen, M., and Downing, T. G. (2008). Beta-N-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. *Ecotoxicol. Environ. Saf.* **71**, 309–313.

Field, N. C., Metcalf, J. S., Caller, T. A., Banack, S. A., Cox, P. A., and Stommel, E. W. (2013). Linking beta-methylamino-l-alanine exposure to sporadic amyotrophic lateral sclerosis in Annapolis, MD. *Toxicon* **70**, 179–183.

Fox, D. A., Grandjean, P., de Groot, D., and Paule, M. G. (2012). Developmental origins of adult diseases and neurotoxicity:

Epidemiological and experimental studies. *Neurotoxicology* **33**, 810–816.

Freischmidt, A., Muller, K., Zondler, L., Weydt, P., Volk, A. E., Bozic, A. L., Walter, M., Bonin, M., Mayer, B., von Arnim, C. A., *et al.* (2014). Serum microRNAs in patients with genetic amyotrophic lateral sclerosis and pre-manifest mutation carriers. *Brain* **137**, 2938–2950.

Garruto, R. M., Gajdusek, C., and Chen, K. M. (1980). Amyotrophic lateral sclerosis among Chamorro migrants from Guam. *Ann. Neurol.* **8**, 612–619.

Guerreiro, R., Bras, J., and Hardy, J. (2015). SnapShot: Genetics of ALS and FTD. *Cell* **160**, 798 e1.

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H. X., *et al.* (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772–1775.

Hilborn, E. D., Roberts, V. A., Backer, L., Deconno, E., Egan, J. S., Hyde, J. B., Nicholas, D. C., Wiegert, E. J., Billing, L. M., Diorio, M., Centers for Disease, C., and Prevention., *et al.* (2014). Algal bloom-associated disease outbreaks among users of freshwater lakes–United States, 2009-2010. *MMWR Morb. Mortal Wkly. Rep.* **63**, 11–15.

Hudnell, H. K. (2008). Cyanobacterial harmful algal blooms: State of the science and research needs. In *Advances in Experimental Medicine and Biology* (N. Back, I. R. Cohen, A. Lajtha, J. D. Lambris, and R. Paoletti, Eds.), Vol. **619**. Springer Science, New York, NY.

Iglesias, A. I., Springelkamp, H., van der Linde, H., Severijnen, L. A., Amin, N., Oostra, B., Kockx, C. E., van den Hout, M. C., van Ijcken, W. F., Hofman, A., *et al.* (2013). Exome sequencing and functional analyses suggest SIX6 is a gene involved in an altered proliferation-differentiation balance early in life and optic nerve degeneration at old age. *Hum. Mol. Genet.* **23**, 1320–1332.

IPCC. (2014). Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (Edenhofer, O., R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, A. Adler, I. Baum, S. Brunner, P. Eickemeier, B. Kriemann, J. Savolainen, S. Schlömer, C. von Stechow, T. Zwickel and J.C. Minx, Eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jiang, L., Eriksson, J., Lage, S., Jonasson, S., Shams, S., Mehine, M., Ilag, L. L., and Rasmussen, U. (2014). Diatoms: A novel source for the neurotoxin BMAA in aquatic environments. *PLoS One* **9**, e84578.

Kabashi, E., Lin, L., Tradewell, M. L., Dion, P. A., Bercier, V., Bourgouin, P., Rochefort, D., Bel Hadj, S., Durham, H. D., Vande Velde, C., *et al.* (2010). Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. *Hum. Mol. Genet.* **19**, 671–683.

Karamyan, V. T., and Speth, R. C. (2008). Animal models of BMAA neurotoxicity: A critical review. *Life Sci.* **82**, 233–246.

Karlsson, O., Berg, A. L., Lindstrom, A. K., Hanrieder, J., Arnerup, G., Roman, E., Bergquist, J., Lindquist, N. G., Brittebo, E. B., and Andersson, M. (2012). Neonatal exposure to the cyanobacterial toxin BMAA induces changes in protein expression and neurodegeneration in adult hippocampus. *Toxicol. Sci.* **130**, 391–404.

Karlsson, O., Bergquist, J., and Andersson, M. (2013). Quality measures of imaging mass spectrometry aids in revealing long-term striatal protein changes induced by neonatal exposure to the cyanobacterial toxin beta-N-methylamino-L-alanine (BMAA). *Mol. Cell. Proteomics.* **13**, 93–104.

Karlsson, O., Lindquist, N. G., Brittebo, E. B., and Roman, E. (2009a). Selective brain uptake and behavioral effects of the cyanobacterial toxin BMAA (beta-N-methylamino-L-alanine) following neonatal administration to rodents. *Toxicol. Sci.* **109**, 286–295.

Karlsson, O., Roman, E., Berg, A. L., and Brittebo, E. B. (2011). Early hippocampal cell death, and late learning and memory deficits in rats exposed to the environmental toxin BMAA (beta-N-methylamino-L-alanine) during the neonatal period. *Behav. Brain Res.* **219**, 310-20.

Karlsson, O., Roman, E., and Brittebo, E. B. (2009b). Long-term cognitive impairments in adult rats treated neonatally with beta-N-Methylamino-L-Alanine. *Toxicol. Sci.* **112**, 185–195.

Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., Burrell, J. R., and Zoing, M. C. (2011). Amyotrophic lateral sclerosis. *Lancet* **377**, 942–955.

Koerner, D. R. (1952). Amyotrophic lateral sclerosis on Guam. *Ann. Intern. Med.* **37**, 1204–1220.

Kovacs, G. G., Adle-Biassette, H., Milenkovic, I., Cipriani, S., van Scheppingen, J., and Aronica, E. (2014). Linking pathways in the developing and aging brain with neurodegeneration. *Neuroscience* **269**, 152–172.

Lage, S., Costa, P. R., Moita, T., Eriksson, J., Rasmussen, U., and Rydberg, S. J. (2014). BMAA in shellfish from two Portuguese transitional water bodies suggests the marine dinoflagellate Gymnodinium catenatum as a potential BMAA source. *Aquat. Toxicol.* **152**, 131–138.

Lemmens, R., Van Hoecke, A., Hersmus, N., Geelen, V., D'Hollander, I., Thijs, V., Van Den Bosch, L., Carmeliet, P., and Robberecht, W. (2007). Overexpression of mutant superoxide dismutase 1 causes a motor axonopathy in the zebrafish. *Hum. Mol. Genet.* **16**, 2359–2365.

Martin, L. J. (2011). Mitochondrial pathobiology in ALS. *J. Bioenerg. Biomembr.* **43**, 569–579.

Masseret, E., Banack, S., Boumediene, F., Abadie, E., Brient, L., Pernet, F., Juntas-Morales, R., Pageot, N., Metcalf, J., Cox, P., French Network on, A. L. S. C. D., and Investigation., *et al.* (2013). Dietary BMAA exposure in an amyotrophic lateral sclerosis cluster from southern France. *PLoS One* **8**, e83406–e83410.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baures, E., and Thomas, O. (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environ. Int.* **59C**, 303–327.

Metcalf, J. S., Banack, S. A., Lindsay, J., Morrison, L. F., Cox, P. A., and Codd, G. A. (2008). Co-occurrence of beta-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990-2004. *Environ. Microbiol.* **10**, 702–708.

Modgil, S., Lahiri, D. K., Sharma, V. L., and Anand, A. (2014). Role of early life exposure and environment on neurodegeneration: Implications on brain disorders. *Transl. Neurodegener.* **3**, 9.

Paerl, H. W., and Paul, V. J. (2012). Climate change: Links to global expansion of harmful cyanobacteria. *Water Res.* **46**, 1349–1363.

Pasinelli, P., and Brown, R. H. (2006). Molecular biology of amyotrophic lateral sclerosis: Insights from genetics. *Nat. Rev. Neurosci.* **7**, 710–723. 10.1038/nrn1971.

Ramesh, T., Lyon, A. N., Pineda, R. H., Wang, C., Janssen, P. M., Canan, B. D., Burghes, A. H., and Beattie, C. E. (2010). A genetic model of amyotrophic lateral sclerosis in zebrafish displays

phenotypic hallmarks of motoneuron disease. *Dis. Model. Mech.* **3**, 652–662.

Reamon-Buettner, S. M., Mutschler, V., and Borlak, J. (2008). The next innovation cycle in toxicogenomics: Environmental epigenetics. *Mutat. Res.* **659**, 158–165.

Reed, D., Labarthe, D., Chen, K. M., and Stallones, R. (1987). A cohort study of amyotrophic lateral sclerosis and parkinsonism-dementia on Guam and Rota. *Am. J. Epidemiol.* **125**, 92–100.

Rodgers, K. J., and Shiozawa, N. (2008). Misincorporation of amino acid analogues into proteins by biosynthesis. *Int. J. Biochem. Cell Biol.* **40**, 1452–1466.

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H. X., *et al.* (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59–62.

Roy, N. M., Arpie, B., Lugo, J., Linney, E., Levin, E. D., and Cerutti, D. (2012). Brief embryonic strychnine exposure in zebrafish causes long-term adult behavioral impairment with indications of embryonic synaptic changes. *Neurotoxicol. Teratol.* **34**, 587–591.

Samson, J. C., Goodridge, R., Olobatuyi, F., and Weis, J. S. (2001). Delayed effects of embryonic exposure of zebrafish (Danio rerio) to methylmercury (MeHg). *Aquat. Toxicol.* **51**, 369–376.

Sasaki, T., and Kishi, S. (2013). Molecular and chemical genetic approaches to developmental origins of aging and disease in zebrafish. *Biochim. Biophys. Acta.* **1832**, 1362–1370.

Sharma, K. R., Kent-Braun, J. A., Majumdar, S., Huang, Y., Mynhier, M., Weiner, M. W., and Miller, R. G. (1995). Physiology of fatigue in amyotrophic lateral sclerosis. *Neurology* **45**, 733–740.

Siddique, T., Figlewicz, D. A., Pericak-Vance, M. A., Haines, J. L., Rouleau, G., Jeffers, A. J., Sapp, P., Hung, W. Y., Bebout, J., McKenna-Yasek, D., Collaborators., *et al.* (1991). Linkage of a gene causing familial amyotrophic lateral sclerosis to chromosome 21 and evidence of genetic-locus heterogeneity. *N. Engl. J. Med.* **324**, 1381–1384.

Simon, N. G., Turner, M. R., Vucic, S., Al-Chalabi, A., Shefner, J., Lomen-Hoerth, C., and Kiernan, M. C. (2014). Quantifying disease progression in amyotrophic lateral sclerosis. *Ann. Neurol.* **76**, 643–657.

Stewart, A., Sandercock, J., Bryan, S., Hyde, C., Barton, P. M., Fry-Smith, A., and Burls, A. (2001). The clinical effectiveness and cost-effectiveness of riluzole for motor neurone disease: A rapid and systematic review. *Health Technol. Assess.* **5**, 1–97.

Swinnen, B., and Robberecht, W. (2014). The phenotypic variability of amyotrophic lateral sclerosis. *Nat. Rev. Neurol.* **10**, 661–670.

Tang, G., Gudsnuk, K., Kuo, S. H., Cotrina, Marisa, L., Rosoklija, G., Sosunov, A., Sonders, Mark, S., Kanter, E., Castagna, C., Yamamoto, A., *et al.* (2014). Loss of mTOR-dependent macroautophagy causes Autistic-like synaptic pruning deficits. *Neuron.* **83**, 1131–1143.

Tartaglione, A. M., Venerosi, A., and Calamandrei, G. (2016). Early-life toxic insults and onset of sporadic neurodegenerative diseases-an overview of experimental studies. *Curr. Top. Behav. Neurosci.* **29**, 231–264.

Testa, D., Lovati, R., Ferrarini, M., Salmoiraghi, F., and Filippini, G. (2004). Survival of 793 patients with amyotrophic lateral sclerosis diagnosed over a 28-year period. *Amyotroph. Lateral. Scler. Other Motor Neuron Disord.* **5**, 208–212.

Torbick, N., Hession, S., Stommel, E., and Caller, T. (2014). Mapping amyotrophic lateral sclerosis lake risk factors across northern New England. *Int. J. Health Geogr.* **13(1)**, 10.1186/1476-072X-13-1.

Trojsi, F., Monsurro, M. R., and Tedeschi, G. (2013). Exposure to environmental toxicants and pathogenesis of amyotrophic lateral sclerosis: State of the art and research perspectives. *Int. J. Mol. Sci.* **14**, 15286–15311.

Vaccaro, A., Patten, S. A., Ciura, S., Maios, C., Therrien, M., Drapeau, P., Kabashi, E., and Parker, J. A. (2012). Methylene blue protects against TDP-43 and FUS neuronal toxicity in C. elegans and D. rerio. *PLoS One* **7**, e42117.

Vahtera, E., Conley, D. J., Gustafsson, B. G., Kuosa, H., Pitkanen, H., Savchuk, O. P., Tamminen, T., Viitasalo, M., Voss, M., Wasmund, N., *et al.* (2007). Internal ecosystem feedbacks enhance nitrogen-fixing cyanobacteria blooms and complicate management in the Baltic Sea. *Ambio* **36**, 186–194.

Vaiserman, A. (2011). Early-life origin of adult disease: Evidence from natural experiments. *Exp. Gerontol.* **46**, 189–192. 10.1016/j.exger.2010.08.031.

Vyas, K. J., and Weiss, J. H. (2009). BMAA–an unusual cyanobacterial neurotoxin. *Amyotroph. Lateral Scler.* **10(Suppl 2)**, 50–55.

Weber, D. N. (2006). Dose-dependent effects of developmental mercury exposure on C-start escape responses of larval zebrafish Danio rerio. *J. Fish Biol.* **69**, 75–94. 10.1111/j.1095-8649.2006.01068.x.

Wirbisky, S. E., Weber, G. J., Lee, J. W., Cannon, J. R., and Freeman, J. L. (2014). Novel dose-dependent alterations in excitatory GABA during embryonic development associated with lead (Pb) neurotoxicity. *Toxicol. Lett.* **229**, 1–8.

Zhang, Y., Schulz, V. P., Reed, B. D., Wang, Z., Pan, X., Mariani, J., Euskirchen, G., Snyder, M. P., Vaccarino, F. M., Ivanova, N., *et al.* (2013). Functional genomic screen of human stem cell differentiation reveals pathways involved in neurodevelopment and neurodegeneration. *Proc. Natl. Acad. Sci. U S A* **110**, 12361–12366. 10.1073/pnas.1309725110.

Zhou, X., Escala, W., Papapetropoulos, S., and Zhai, R. G. (2010). beta-N-methylamino-L-alanine induces neurological deficits and shortened life span in Drosophila. *Toxins* **2**, 2663–2679.

Zufiria, M., Gil-Bea, F. J., Fernandez-Torron, R., Poza, J. J., Munoz-Blanco, J. L., Rojas-Garcia, R., Riancho, J., and de Munain, A. L. (2016). ALS: A bucket of genes, environment, metabolism and unknown ingredients. *Prog. Neurobiol.* **142**, 104–129.