



The Effects of Long-term, Low-dose β -N-methylamino-L-alanine (BMAA) Exposures in Adult SOD^{G93R} Transgenic Zebrafish

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Abstract

β -N-Methylamino-L-alanine (BMAA) is a non-proteinogenic amino acid produced by cyanobacteria, which has been implicated in several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). It is postulated that chronic exposure to BMAA can lead to formation of protein aggregates, oxidative stress, and/or excitotoxicity, which are mechanisms involved in the etiology of ALS. While specific genetic mutations are identified in some instances of ALS, it is likely that a combination of genetic and environmental factors, such as exposure to the neurotoxin BMAA, contributes to disease. We used a transgenic zebrafish with an ALS-associated mutation, compared with wild-type fish to explore the potential neurotoxic effects of BMAA through chronic long-term exposures. While our results revealed low concentrations of BMAA in the brains of exposed fish, we found no evidence of decreased swim performance or behavioral differences that might be reflective of neurodegenerative disease. Further research is needed to determine if chronic BMAA exposure in adult zebrafish is a suitable model to study neurodegenerative disease initiation and/or progression.

Keywords L-BMAA · D-BMAA · AEG · DAB · ALS · Zebrafish · Neurotoxicity · Neurodegeneration · SOD1

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with an estimated prevalence of greater than seven in one hundred thousand Americans (Mehta 2017). The average age at diagnosis is 55 years old, with a range of 40 to 70 years old (The ALS Association, <https://www.als.org/understanding-als>). ALS is characterized by death of motor neurons (MNs), which manifests in progressive loss of muscle control leading to paralysis, inability to speak,

and eventually respiratory failure. Familial ALS (fALS), or those cases that can be linked directly to an inherited genetic mutation, accounts for approximately 10% of cases (Mulder et al. 1986). Consequently, the remaining 90% of cases are of unknown origin and are classified as sporadic (sALS). Fortunately, many similarities exist between the presentation of disease pathologies in both forms of ALS, which makes exploration of mechanisms applicable across both fALS and sALS (Rosen et al. 1993).

It is postulated that genetic differences can predispose some individuals to disease given the appropriate environmental stimulus (Price et al. 2015; Eichler 2019; Sher 2017). To date, multiple mutations responsible for fALS have been discovered, but this does not address the broader genetic variability inherent among all individuals in a population. This led us to speculate that a combination of genetic and environmental factors may act concomitantly to initiate and/or exacerbate disease in many instances of ALS.

We investigated potential gene-by-environment effects related to incidence of ALS through the study of β -N-methylamino-L-alanine (BMAA), a neurotoxin found worldwide in terrestrial, marine, and freshwater environments (Réveillon et al. 2015; Chiu et al. 2011; van Onselen et al.

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2018). It is produced by cyanobacteria, the bacteria responsible for blue-green algal blooms (Wiltse et al. 2018). Research shows that harmful cyanobacterial blooms are increasing because of eutrophication and climate change (Neil et al. 2012), eliciting an urgency to decipher the role of cyanotoxins in neurodegenerative diseases. BMAA, one of many cyanotoxins produced by bacteria, was first associated with ALS on the Island of Guam following World War II when it was discovered that the indigenous Chamorro people experienced ALS/Parkinsonism dementia complex (ALS/PDC) at rates 50–100 times higher than the US mainland rates for ALS (Cox et al. 2003). This was linked to ingestion of BMAA-laced seeds of the cycad tree, a seed used to produce flour for the Chamorro diet, as well as through the consumption of fruit bats and pigs that fed on the seeds (Banack et al. 2006). It was demonstrated that cyanobacteria living in a symbiotic relationship within specialized roots of the cycad tree produce the neurotoxin, BMAA (Murch et al. 2004a). Additionally, it was discovered that BMAA biomagnifies in the seed, then in each subsequent trophic level, and ultimately in people, where it was seen in post-mortem brain samples from Chamorro people who died from ALS/PDC, but not generally in control tissues (Murch et al. 2004b).

Higher than normal rates of ALS have been reported in other locations globally where potential exposure to high concentrations of BMAA exists. Some examples are areas near lakes in New Hampshire with toxic algal blooms (Caller et al. 2009); regions of Sweden, France, and Florida where BMAA has been isolated in shellfish (Jiang et al. 2015; Masseret et al. 2013; Brand et al. 2010); and areas with high concentrations of BMAA in the desert dust where US veterans previously served during Operation Desert Storm (Cox et al. 2009). Additionally, high concentrations of BMAA were isolated in the brains of 13 of 14 stranded dolphins from Florida and Massachusetts in which elevated levels of β amyloid plaques and dystrophic neurites were observed (Davis et al. 2019).

The possible role of BMAA in neurodegenerative disease has been studied extensively. BMAA acute exposures in rodents and chicks (Vega et al. 1968), as well as in macaques (Spencer et al. 1987), generated behavioral disorders, while BMAA exposures in honeybees resulted in impaired learning ability (Okle et al. 2013). Exposure in pregnant and neonatal mice revealed hyperactivity in open field tests, balance problems (impaired locomotion), and thigmotaxis (Karlsson et al. 2009a). Other studies have demonstrated maternal transfer of BMAA to offspring, in both mice (Andersson et al. 2013) and birds (Andersson et al. 2018; Kim and Rydberg 2020), as well as altered metabolism and signaling pathways in the hippocampus of mice (Karlsson et al. 2012). Additionally, the role of BMAA in initiation and progression of ALS is supported by experimental evidence in non-human primates; however, much remains unknown. Chronic BMAA exposure in vervets produced neurofibrillary tangles and beta-amyloid plaques in brains commensurate of

Guam ALS/PDC (Cox et al. 2016), while more recent work expands on previous findings revealing ALS/motor neuron disease-like phenotypes following chronic BMAA exposure in adults, including upper and lower motor neuron degeneration, characteristic pathological responses in glial cells, and proteinopathies including TDP-43 aggregation and ubiquitinated insoluble inclusions (Davis et al. 2020).

We examined adult zebrafish to study the potential effects of BMAA in a vertebrate system with an easily interrogated nervous system (Babin et al. 2014). Increasingly, zebrafish have emerged as a viable model to probe a variety of research questions (Khan and Alhewairini 1989). This small fish indigenous to southeast Asia shares roughly 70% conservation with the human genome (Santoriello and Zon 2012), and 82% of human disease genes have orthologs in the fish (Langheinrich 2003). While sexually productive for up to 2 years, zebrafish can live three or more years offering opportunity to study long-term effects of early-life toxicological or pharmaceutical exposures, as well as age-dependent disorders (Santoriello and Zon 2012).

To explore potential gene-by-environment interactions, we leveraged *sod1* mutant zebrafish to study chronic long-term BMAA exposures. Previous work (Ramesh et al. 2010) showed that *sod1*^{G93R} (one of the most common of more than 150 *SOD1* ALS mutations) transgenic fish displayed phenotypes including MN loss and muscle pathology indicative of ALS. Characterization of this transgenic fish showed a decrease in endurance in a swim tunnel assay and neuromuscular junction changes in adult fish. Here, we used this fish to determine if neurotoxic exposure accelerates disease phenotypes in a model organism with a known ALS mutation.

To date, most published work on zebrafish related to BMAA has relied on developmental exposures to explore potential neurotoxic effects (Roy et al. 2017; Frøyset et al. 2016; Powers et al. 2017). While this has permitted insight into BMAA's influences on the developing nervous system and possible consequences in later life, it fails to address the role that adult chronic exposure to BMAA plays in neurodegeneration. We therefore focused our attention on long-term chronic BMAA exposures based on the premise that although developmental exposure to BMAA may play a role in disease (Engskog et al. 2013; Karlsson et al. 2009b; Scott and Downing 2017), chronic exposure to low concentrations of a toxicant or other harmful agents may also be important (Cox et al. 2016; Davis et al. 2020).

Materials and Methods

Zebrafish Husbandry

AB and *Tg(sod1:sod1G93R;hsp70l:DsRed)os10* (hereafter referred to as *sod1*^{G93R}) (Ramesh et al. 2010) fish lines were maintained at 28 °C with a 14/10-h light/dark cycle. Adult

fish were fed dry fish flake food (TetraMin Tropical Flakes, Spectrum Brands, Inc., Germany) twice daily. Embryos were reared in 0.5×E2 media (7.5 mM NaCl (Fisher Scientific, Fair Lawn, NJ, BP358-212), 0.25 mM KCl (Sigma-Aldrich, St. Louis, MO, P9541), 0.5 mM MgSO₄ (Sigma, M-9397), 75 µM KH₂PO₄ (Sigma, P0662), 25 µM Na₂HPO₄ (VWR International, West Chester, PA, BDH4540), 0.5 mM CaCl₂ (Sigma, C-3881), 0.35 mM NaHCO₃ (Fisher, BP328-500), 0.5 mg/L methylene blue (Sigma, M9141)) with daily media changes until 5 days post-fertilization (dpf) at 28.5 °C with a 14/10-h light/dark cycle. Larvae were then housed in 500 mL of fish system water (with 500 mL added every 2 days until full) in 3-L tanks on a heat pad with a water temperature of 28 °C with a 14/10-h light/dark cycle for approximately 2 weeks. Larvae were fed larval fish food (Larval AP100-2 Dry Larval Diet, Zeigler Bros, Inc, USA) beginning at 5 dpf. All fish husbandry, anesthesia, and euthanasia were conducted in accordance with NC State Institutional Animal Care and Use Committee (IACUC)-approved protocols.

BMAA

L-BMAA hydrochloride (50 mg, CAS number 16012–55-8, MW 154.60) was purchased from Sigma-Aldrich and reconstituted with Milli-Q (18 MΩ) water to a stock concentration of 100 mM. 500-µL aliquots were stored at –20 °C until needed. Working concentrations were made fresh with 100 mM BMAA diluted to the desired concentration in system fish water.

Spawning and BMAA Exposures

Wild-type (WT) AB fish were crossed with *sod*^{G93R} fish. Larvae were screened for dsRed expression with a Leica MZ FLIII fluorescence stereomicroscope at four dpf to segregate larvae with dsRed expression, classified as *sod*^{G93R} fish, from clutch mates without dsRed expression, classified as WT fish. BMAA exposure began at 3 weeks post-fertilization (wpf) with weekly water changes.

Long-term Exposure Diets

Fish were fed daily for two and one-half years with either a control or a 100-µM BMAA diet. Solid larval fish food (water stable, <50 microns particle size) was mixed in fish system water and used as a vehicle for the neurotoxin exposure. Food was prepared twice per week by mixing 2.0 mg/mL larval fish food in system fish water for control fish and 2.0 mg/mL larval fish food in system fish water spiked with 100 mM BMAA for a final concentration of 100 µM BMAA. Fish were fed 4 mL of this diet daily per 3-L tank, with juvenile and adults being supplemented with dry adult flake food

twice daily. The number of fish per tank ranged from 10 to 40; therefore, the amount eaten daily by each fish varied.

Light–Dark Behavioral Assay

A light–dark behavioral assay was conducted using the DanioVision box with EthoVision® XT software (Noldus; Leesburg, VA, USA). Larval behavior was assessed at 6 dpf with larvae arrayed in a 96-well plate, one larva per well, and placed within the DanioVision box. Following a 10-min dark acclimation period, individual larva movement was recorded over five light–dark cycles lasting 10 min per cycle.

Novel Tank Assay

A novel tank assay was conducted based on a previously described protocol (Cachat et al. 2010). Individual fish were taken from a 3-L group tank and put into a novel 1-L tank where their movement was tracked over a 5-min period beginning immediately after introduction into the arena. An f = 4–8 mm F1.4 CCTV lens (Computar Mega-Pixel, H2Z0414C-MP) and EthoVision XT software were used to record activity of individual fish introduced into the novel tank. A heat map of fish movement within the arena was generated for each fish, and total distance moved was plotted for individuals within one of four treatment groups. Statistical analysis was conducted using one-way ANOVA.

Swim Beaker Assay

A swim beaker assay was carried out in a 1000-mL beaker with a 9×50 mm stir bar in 800 mL of fish water based on a previously described protocol (Powers et al. 2017). Briefly, individual fish were introduced into the beaker, which was resting on a magnetic stirrer within a cardboard box to minimize external visual stimulation, and allowed to acclimate for 2 min. Stirrer was turned on and brought up to 300 rpm within 20 s. Fish were allowed to swim until they were no longer able to maintain position against the current for a period of 30 s. A cutoff time of 300 s was arbitrarily set. The swim endurance of each fish was measured in three sessions, with 30-s rest periods between sessions. The mean time of the three measurements was plotted for each individual by treatment. Statistical analysis was performed using Graph-Pad Prism software (La Jolla, CA, USA) using the log-rank (Mantel-Cox) test (*p*-value alpha of 0.05).

Swim Tunnel Assay

A Loligo System swim tunnel (5–185 L) with an SEW-Eurodrive 60 Hz motor and SEW-Eurodrive 0.5 HP/0.37 kW Movitrac LTE controller (Loligo Systems, Viborg, Denmark) was used to analyze swim endurance. Up to 11 fish per trial

were loaded in the swim chamber with a starting water temperature of 25.5 °C ($\pm 1^\circ$) and allowed to acclimate for 20 min at a flow rate of 2 cm/s. Following the acclimation period, motor output was adjusted to 10 Hz, representing a flow rate of 13 cm/s, and increased by 2 Hz every 2 min up to a maximum of 60 Hz (123 cm/s). Fish were allowed to swim until fatigued and unable to maintain their position in the chamber, at which point they impinged upon the back wire mesh of the swim chamber and were immediately removed. Time spent in the swim tunnel was recorded and individual fish were subsequently imaged and measured, and their sex recorded. All tests were performed blind. Swim time was plotted with a Kaplan–Meier curve and a log-rank test was used to test for differences in the swim time of fish from different treatment groups at an alpha level of 0.05. To test whether covariates such as length of fish, temperature, or day have an effect on the length of time spent in the swim tunnel, we used a Cox proportional-hazards regression model. Finally, ANOVA was performed to determine any gene, environment, or gene-by-environment effect. We confirmed normality in the data and homogeneity of variance using QQ and residual plots respectively, before carrying out ANOVA.

Brain Tissue Dissection

After two and one-half years of BMAA exposure, long-term BMAA-exposed (WT and *sod*^{G93R}) and control zebrafish (WT and *sod*^{G93R}) were euthanized with 4% Tricaine-S (Syndal, Ferndale, WV) in PBS diluted 1:40 in Milli-Q water. Brains were dissected from fish, immediately flash frozen in liquid nitrogen, and stored at -80°C .

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) Mass Spectrometry Analysis

In preparation for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis, frozen brain tissue (10 per treatment condition) was weighed and homogenized by sonication (three times for 5 s each at 5 W on ice) in 10% trichloroacetic acid (TCA, CAS 76–03-9, Sigma-Aldrich, St. Louis, MO). Samples were left to precipitate at 4 °C for 12–16 h (overnight) after which they were centrifuged ($14,000 \times g$ for 3 min) and the supernatant was removed. A second aliquot of TCA was added followed by sonication, 2-h precipitation at room temperature (RT), centrifugation, and removal of the supernatant. Supernatants were pooled and centrifuged in centrifugal filter tubes (Ultrafree-MC GV 0.22 μM , Sigma-Aldrich), and then diluted 1:20 in Direct-Q® 3 UV (DQ) water (Millipore filtration system Millipore Sigma, Burlington, MA). Diluted samples, along with five serially diluted BMAA standards, were derivatized with AQC (a compound that reacts with primary and secondary amines to improve detection by mass

spectrometry, WAT052880, Waters Corp, Milford, MA) in 20 μL standard/sample + 58 μL 0.2 M borate buffer with 2 μL internal standard $\text{D}_3^{15}\text{N}_2$ -BMAA (concentration 76.4 ng/mL, Isotec Inc., Miamisburg, OH) + 20 μL AQC. Samples were analyzed using a validated method in a Thermo TSQ Quantiva triple quadrupole mass spectrometer in positive ion mode (Banack 2021). BMAA, AEG, and DAB peak identity were confirmed by comparison with commercial reference standards (BMAA-CAS 16012–55-8 (Sigma-Aldrich), DAB-CAS 1883–09-06 (Sigma-Aldrich); AEG-CAS 24123–14-6 (TCI America, Portland, OR)) and BMAA retention time was verified using the stable isotope internal BMAA standard. Quantification was calculated using a 4-point calibration curve (range 0.037–2.4 ng/mL, $R^2 > 0.99$) relating peak area to concentration. The instrument limit of detection was 0.01 ng/mL, and the lower limit of quantification was 0.037 ng/mL.

N-(4-Nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester (NIFE) Enantiomer Mass Spectrometry

NIFE (sc-253524, Santa Cruz Biotechnology, Callas, TX) derivatization of standards for *L*-BMAA, *D*-BMAA (gift from P.B. Wyatt), *N*-(2-aminoethyl) glycine (AEG), *L*-2,4-diaminobutyric acid (DAB), and *D*-DAB (gift from P.B. Nunn) were prepared as follows: 12.5 μL 0.2 M sodium tetraborate, 6.25 μL standard (1 $\mu\text{g/mL}$), and 6.25 μL NIFE were incubated together for 20 min at RT, and then mixed with 2.5 μL 4 M HCl and 97.5 μL DQ water. NIFE-derivatized standards were diluted 1:50, 1:100, 1:200, and 1:300. Undiluted, filtered supernatant from tissue samples in previous analysis was NIFE-derivatized as follows: 10 μL sample, 1.5 μL 4 M NaOH, 28.8 μL sodium tetraborate, and 6.25 μL NIFE were incubated together for 20 min at RT. To this was added 5 μL 4 M HCl and 75.5 μL DQ water. Samples were analyzed using the same Thermo TSQ Quantiva triple quadrupole mass spectrometer from previous analysis with a Thermo Scientific hypersil gold reversed-phase C18 column (25,002–102,130, 100 mm, 2.1 mm ID, 1.9 μM particle size) at 3500 V in positive ion mode. Settings were static spray voltage, sheath gas at 20 Arb (arbitrary unit), aux gas at 2 Arb, sweep gas at 1 Arb, ion transfer tube at 350 °C, vaporization temperature at 400 °C, cycle time of 0.3 s, Q1 resolution at 0.7 FWHM, Q3 at 0.7 FWHM, and CID (collision induced dissociation) nitrogen gas at 1.5 mTorr (for the second quadrupole, which is a vacuum chamber). Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were used for an 8-min gradient curve (0.2 mL/min) with the following parameters: 37% B curve 5; 1.0 min, 58% B curve 9; 5.1 min, 58% B curve 9; 5.5 min, 100% B curve 5; 5.6 min 37% B curve 5; 8 min 37% B curve 5. Quantification was calculated from authenticated

standards using a 4-point calibration curve within a linear range (0.166–0.98 ng/mL, $R^2 > 0.99$).

Hydrolyzed Protein Pellet

Protein pellets from homogenized and centrifuged brain tissue samples were transferred to glass vials, washed, and vortexed two times in 6 M HCl, and heated to 110 °C for 16 h. After removal from heat, samples were vortexed. 100 µL was removed and centrifuged in centrifugal filter tubes (Ultrafree-MC GV 0.22 µm) at 14,000×g for 3 min. Samples were diluted 1:50 in DQ (18 MΩ) water, AQC derivatized, and analyzed by MS per above protocol.

Fish Food Analysis

The fish food was tested for BMAA concentrations. Dried fish flakes were weighed, and 20% TCA was added (0.22 mg/µL). Liquid samples of BMAA-treated larval fish food were stored at 4 °C and extracted 27 days after preparation and mixed with an equal volume of 20% TCA. All samples were vortexed and sonicated (3 W) for 30 s and precipitated at room temperature for 2 h. The sample was then centrifuge/filtered (Ultrafree-MC GV 0.22 µm, Sigma-Aldrich) at 14,000×g for 2 min and derivatized using 6-aminoquinolyl-N hydroxysuccinimidyl (AQC) carbamate derivatization (20 µL sample + 58 µL 0.2 M borate buffer + 2 µL internal standard D₃ ¹⁵N₂-BMAA, concentration 76.4 ng/mL + 20 µL AQC). All samples were also hydrolyzed overnight at 110 °C after first being mixed with HCl. Liquid samples had an equal volume of 12 M HCl added, to measure total BMAA, which is both free and any potential protein-bound BMAA present. The protein pellet from the TCA extraction was taken from the dried fish food flakes and 1 ml of 6 M HCl was added. Samples underwent centrifugal filtration and derivatization as above. Samples were analyzed with mass spectrometry following Banack (Banack 2021). A 7-point standard curve ($R^2 = 0.99$) and appropriate blanks (water, derivatized AQC, HCl) were analyzed alongside the samples for quality control and quantification.

Results

Adult Long-term BMAA Exposures

Wild-type fish (AB strain) were crossed with *sod*^{G93R}, and embryos were sorted based on dsRed fluorescent expression into two groups: fluorescent embryos were classified as *sod*^{G93R} and non-fluorescent embryos were classified as WT, as previously described (Ramesh et al. 2010) (Fig. 1a). Daily exposure to 100 µM BMAA was initiated

at 3 weeks post-fertilization, with fish distributed into one of four treatment groups, *sod*^{G93R}, *sod*^{G93R} + BMAA, WT, or WT + BMAA. Three weeks following the start of exposures, fish were subjected to a light–dark assay. Results from three separate trials showed no consistent behavioral phenotype in *sod*^{G93R} or WT BMAA-exposed fish (Fig. 1b–g).

After the first year of exposure, individual fish from all treatment groups were evaluated in a novel tank assay (Fig. 2a). Initially, *sod*^{G93R} + BMAA fish showed significant difference in behavior from the other treatment conditions. This finding was not reproducible, however, with additional trials showing inconsistent results (Fig. 2b). A swim beaker endurance assay revealed no differences between BMAA-exposed and unexposed fish within each genotype (WT and *sod*^{G93R}) (Fig. 3a). Interestingly, significant differences were seen in swim endurance between genotypes, for both exposed and unexposed fish, further validating the suitability of the *sod*^{G93R} model (Fig. 3b). Follow-up swim tunnel (Supplemental Video 1) trials at almost 3 years of age recapitulated the results seen in previous swim beaker trials (Fig. 4). Pairwise log-rank test showed no significant differences as a result of BMAA exposure, but significant differences were observed in swim endurance between genotypes for both exposed and unexposed groups. Length of fish and temperature did not vary significantly across treatment groups (Cox regression p -value > 0.05); hence, the Cox regression analysis and log-rank test showed similar results. Type II ANOVA revealed no gene-by-environment (p -value = 0.76) or environmental (p -value = 0.32) effects but did indicate a genetic effect (p -value = 0.0008).

Mass Spectrometry Analysis

Blinded UPLC-MS/MS analysis revealed free BMAA in both WT + BMAA and *sod*^{G93R} + BMAA brain tissue, while no BMAA was detected in control WT or *sod*^{G93R} fish brain tissue. Analysis of the brain-derived hydrolyzed protein pellet showed no protein-bound BMAA above the limit of detection (0.01 ng/mL) (Table 1). NIFE derivatization of samples detected the *L* enantiomers of BMAA and DAB, but not the *D* enantiomers of either BMAA or DAB (Table 2). BMAA was confirmed in the liquid diet (Table 3). All the BMAA should have been in a free form, and this was confirmed by comparing the total BMAA hydrolysis sample to the TCA extracted sample which revealed no substantial difference (Table 3). No BMAA was present in the system water used to prepare the liquid diet. Analysis of the tank water before feeding indicated a small BMAA peak that could be residual carry-over from prior use of the tank itself.

Fig. 1 Three-week BMAA exposure behavioral assays. **a** Image of fish expressing dsRed fluorescence. **b–g** Exposures began at 21 dpf. Assays were performed in six-well plates with four fish per well and tracked by Noldus EthoVision software. Graphs display average distance moved per fish in alternating light, dark cycles following a 10-min acclimation period. Error bars represent SEM. Three successive trials showed inconsistent results in *sod*^{G93R} transgenic fish **b–d**, with inconsistent results also seen in WT fish **e–g**

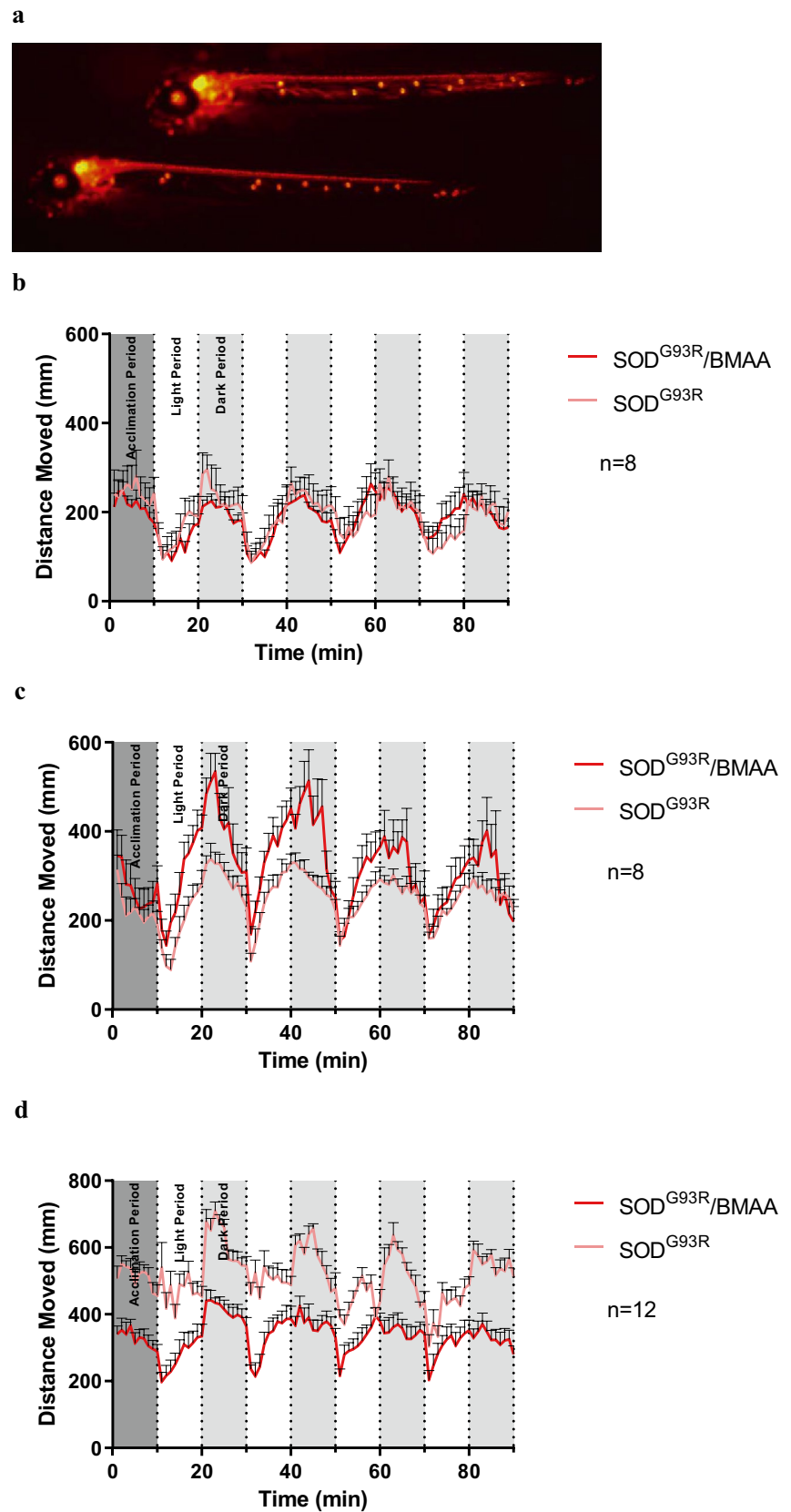
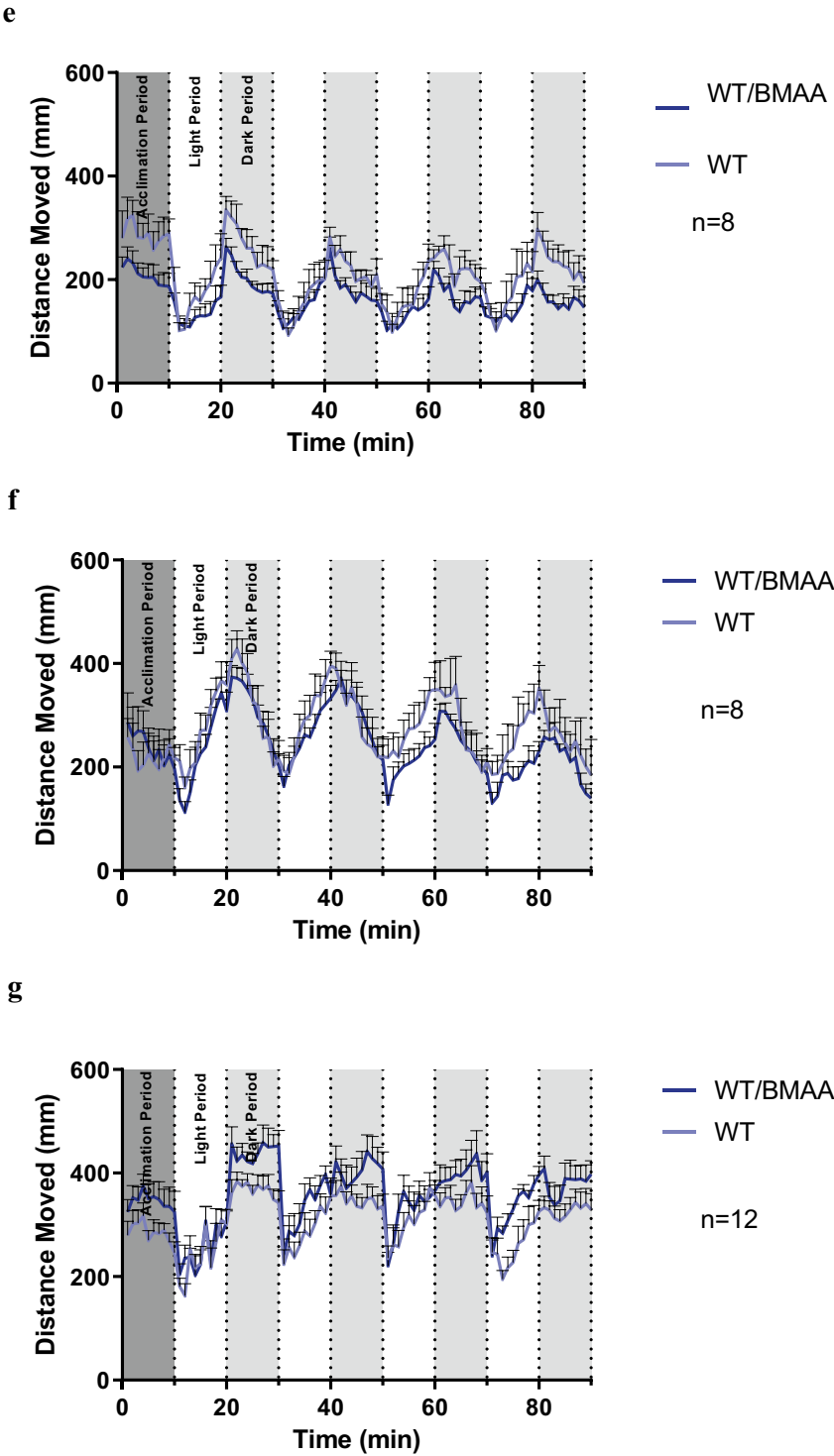


Fig. 1 (continued)



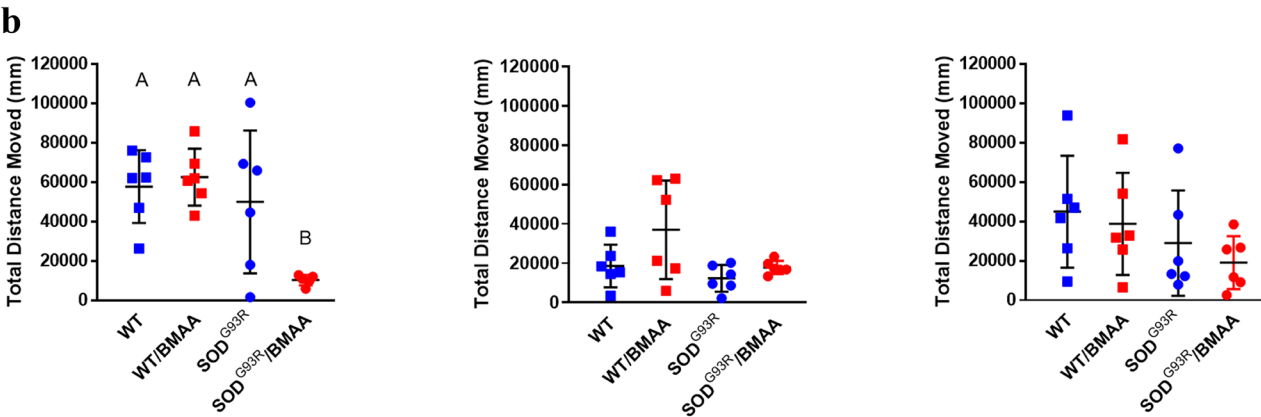
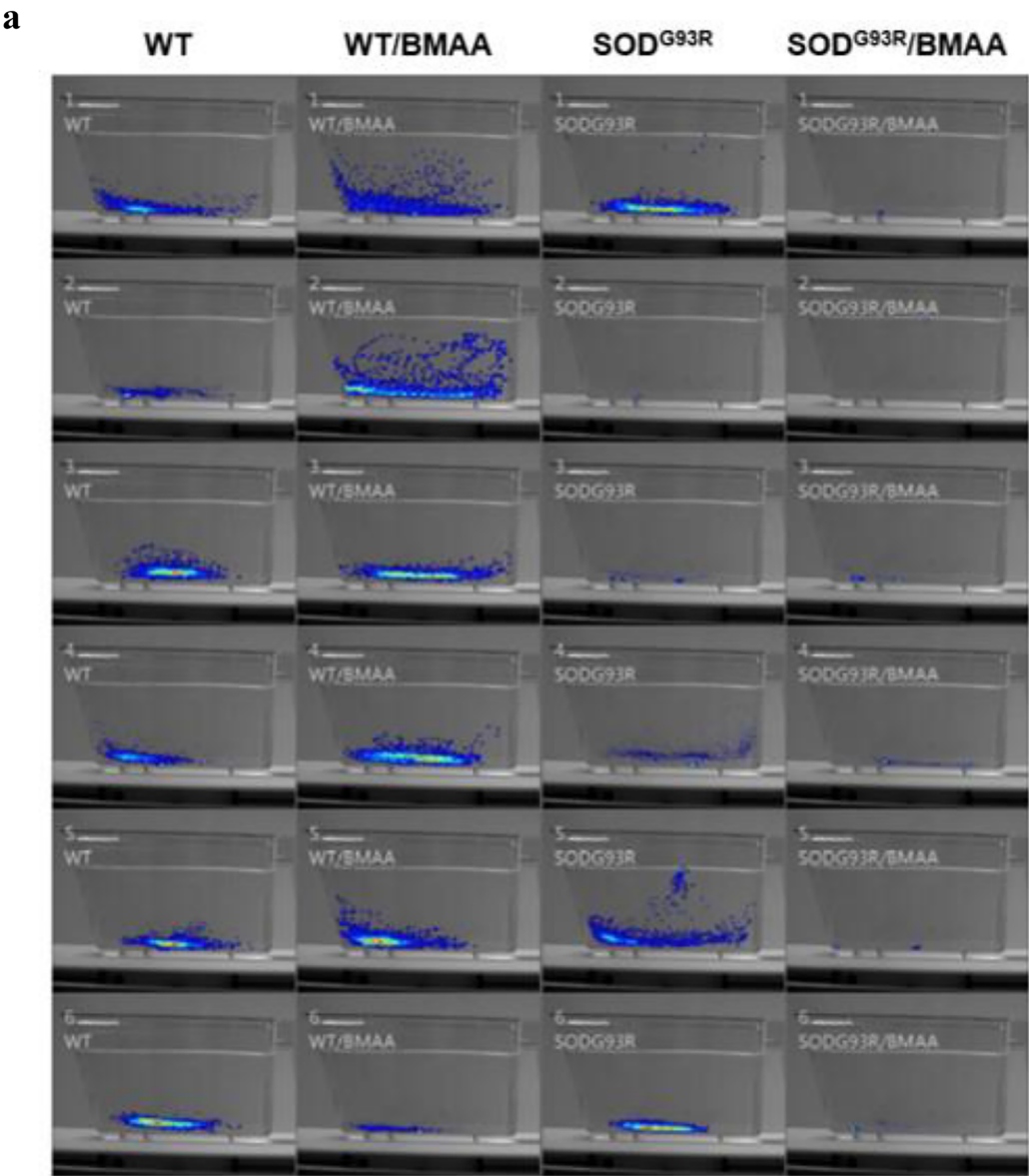


Fig. 2 Novel tank assay in long-term BMAA-exposed fish. One-year old fish that were group housed in a 3-L tank were individually introduced into a novel 1-L tank. Fish movement was tracked for 5 min starting immediately upon introduction into tank by a camera that was linked to a computer utilizing Noldus EthoVision software. Following completion of recording, the fish was removed from the tank and another fish was introduced into the tank and recorded. **a** Heat maps of individual fish movement (the spectrum of colors progressing from dark blue to red represents increasing lengths of time spent in an area). Heat map from only one trial is shown as an example. Results in subsequent trials were inconsistent, as evidenced by quantification in next panel. **b** Total distance moved for each of six fish in each treatment group. Results shown from three separate trials. Different letters indicate significant differences between means ($p < 0.05$) based on one-way ANOVA

Discussion

Since the discovery of high rates of ALS-like disease on the island of Guam more than half a century ago, the potential etiological roles of the neurotoxin BMAA and its structural isomers AEG and DAB have garnered much attention (Murch et al. 2004a; Regueiro et al. 2017; Tan et al. 2018; Karlsson et al. 2014; Mondo et al. 2012). BMAA exposure has been studied extensively in vitro and in vivo in multiple cell lines and various model organisms (Karlsson et al. 2009a; Cox et al. 2016; Wang et al. 2020). Additionally, cyanotoxin mixtures of BMAA, AEG, and DAB, among others, have been analyzed to determine possible synergistic effects of multiple naturally occurring toxins to more closely mirror probable environmental exposures (Martin et al. 2019, 2021). While many mechanisms have been proposed by which BMAA may initiate and/or exacerbate disease (Roy et al. 2017; Chiu et al. 2012; Dunlop et al. 2013; Lindwall and Cole 1984; Neumann et al. xxxx; Nunn and Ponnusamy 2009; van Onselen and Downing 2018), much remains unknown. Here, we chose to examine the role of chronic BMAA exposure only, as this is an area of potential BMAA-induced pathology that is unexplored in aged zebrafish adults. Our work involved investigating BMAA exposures in a vertebrate model to identify potential phenotypes and study underlying mechanisms associated with chronic exposure throughout the life course. We explored the possible gene-by-environment component of neurodegenerative disease by utilizing a transgenic fish harboring a familial ALS mutation (Ramesh et al. 2010).

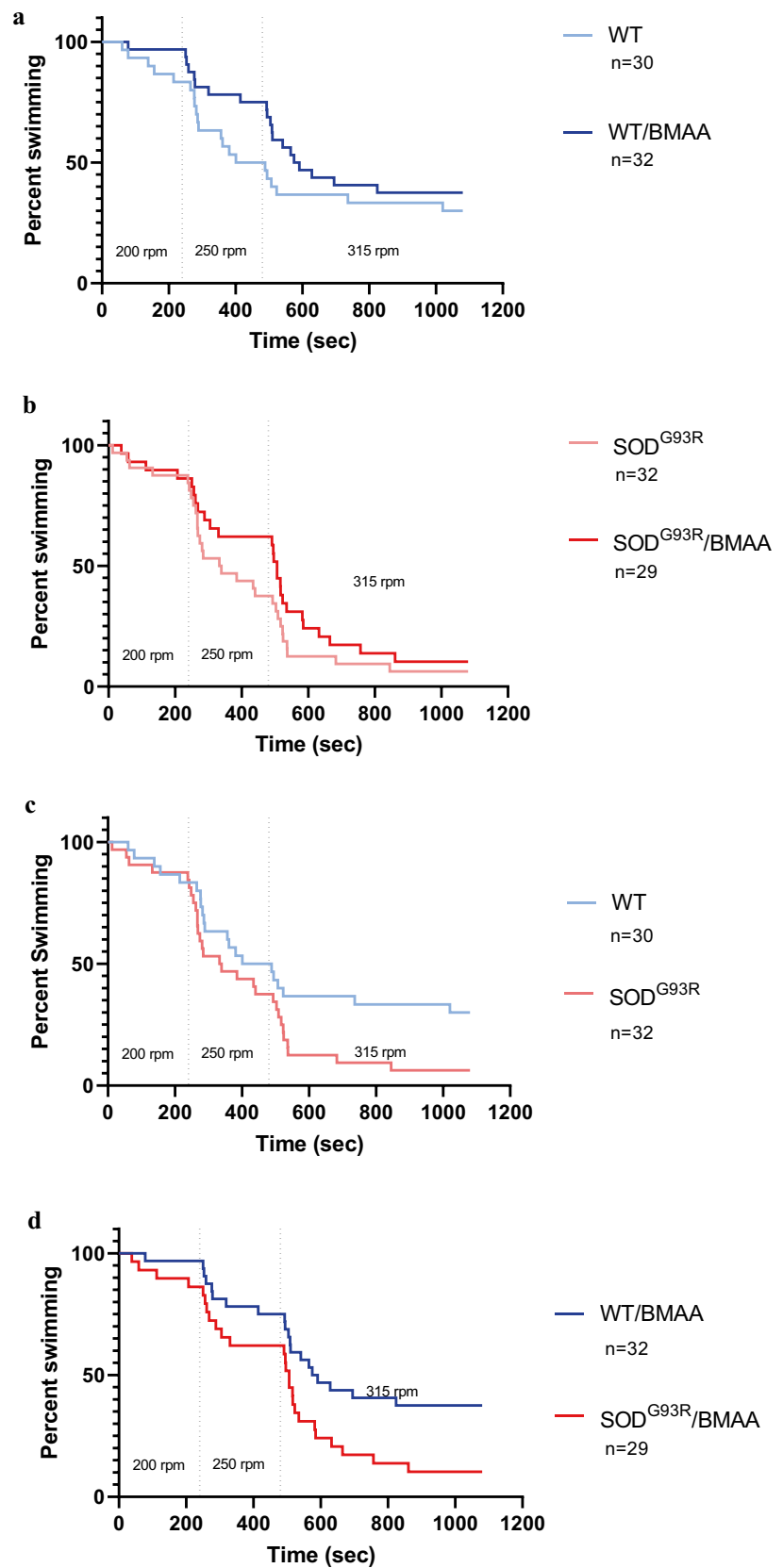
BMAA exposures in the long-term study were started at 3 weeks post-fertilization to avoid the organogenesis window in zebrafish (Ackermann and Paw 2003). We chose to expose the fish to a 100- μ M BMAA diet, based on our work (previously, we had conducted short-term dose response assays in zebrafish larvae with concentrations from 10 μ M to 50 mM) and that of others (Powers et al. 2017; Beri et al. 2017), believed to be consistent with potential environmental exposure and developmental effects, but low enough to

be considered chronic. Because zebrafish are opportunistic feeders and readily consume food presented (Kolb et al. 2018), it was determined that incorporating BMAA into their food source was likely the most reliable method of exposure. Additionally, incorporating BMAA only in the tank water to the desired concentration would require prohibitive amounts of BMAA to be used. Immediate consumption was important because once allowed to dilute in the tank water, the BMAA concentration dropped from 80 μ M in the diet (per UPLC-MS/MS analysis) to 0.2 μ M in the tank water if unconsumed. This was confirmed by MS analysis of the tank water 20 min post-feeding (Table 3, 0.03 μ g/mL = 0.194 μ M). MS analysis of brain tissue extracted from adult fish confirmed the presence of free BMAA in exposed fish (mean 26 ng/g, compared with brain tissue from Guam Chamorro individuals with average levels of ~6800 ng/g¹⁶ and free-living carp brains with 430 ng/g⁶²) with controls showing no free BMAA. Protein-bound BMAA was not detected in either group (Table 1), whereas protein-bound BMAA was detected in brains (mean 0.32 mg/g) of low-dose exposed vervets (Cox et al. 2016).

These exposures were conducted over a period of two and one-half years with no abnormal phenotypes observed during this period. In contrast to our findings, lower doses of BMAA in this same transgenic *sod*^{G93R} line were reported to negatively impact nerve length and neuromuscular junction colocalization in larvae, and swim endurance and fatigability in 5-month-old adults (Powers et al. 2017). Previous immunohistochemistry assays in our lab showed no evidence of post-synaptic acetylcholine receptor or muscle actin filament changes at 5 mM BMAA exposure in 48 hpf embryos (data not shown), while work by Martin et al. revealed no aberrant locomotor behavior in WT Tüpfel longfin larvae exposed to 1000 μ M BMAA (Martin et al. 2021). While these findings support our observed lack of behavioral impairments resulting from BMAA exposure, swim beaker and swim tunnel assays revealed swim endurance differences between WT and *sod*^{G93R} transgenic fish independent of exposure to BMAA (Figs. 3 and 4), demonstrating that swim performance differences were detectable, and supporting previous characterization of this fish line (Ramesh et al. 2010).

It has been previously established that BMAA is able to cross the blood–brain barrier (Duncan et al. 1991; Smith et al. 1992). To determine if BMAA was consumed at sufficient concentrations for transport to the brain of exposed fish, UPLC-MS/MS was used to investigate the presence of free BMAA, protein-bound BMAA, and enantiomers of BMAA in brain tissue. Additionally, MS was also used to detect and quantify any AEG or DAB present. The identification of free BMAA in both WT exposed and *sod*^{G93R} exposed fish, but not in controls, verified the effectiveness of our experimental feeding model. However, the lack of protein-bound BMAA suggests a different mechanism in

Fig. 3 Swim beaker assay for long-term BMAA exposures. A swim endurance trial with increases in rpm at 4 min and 8 min was conducted for individual fish. **a, b** Graphs representing impact of BMAA exposure across two genotypes. **c, d** Graphs representing differences in swimming endurance by genotype. Study was conducted blind with resulting log-rank (Mantel-Cox) test *p*-values of 0.2569, 0.1978, 0.0375, and 0.0159, respectively. WT, light blue; WT+BMAA, dark blue; *sod*^{G93R}, light red; *sod*^{G93R}+BMAA, dark red



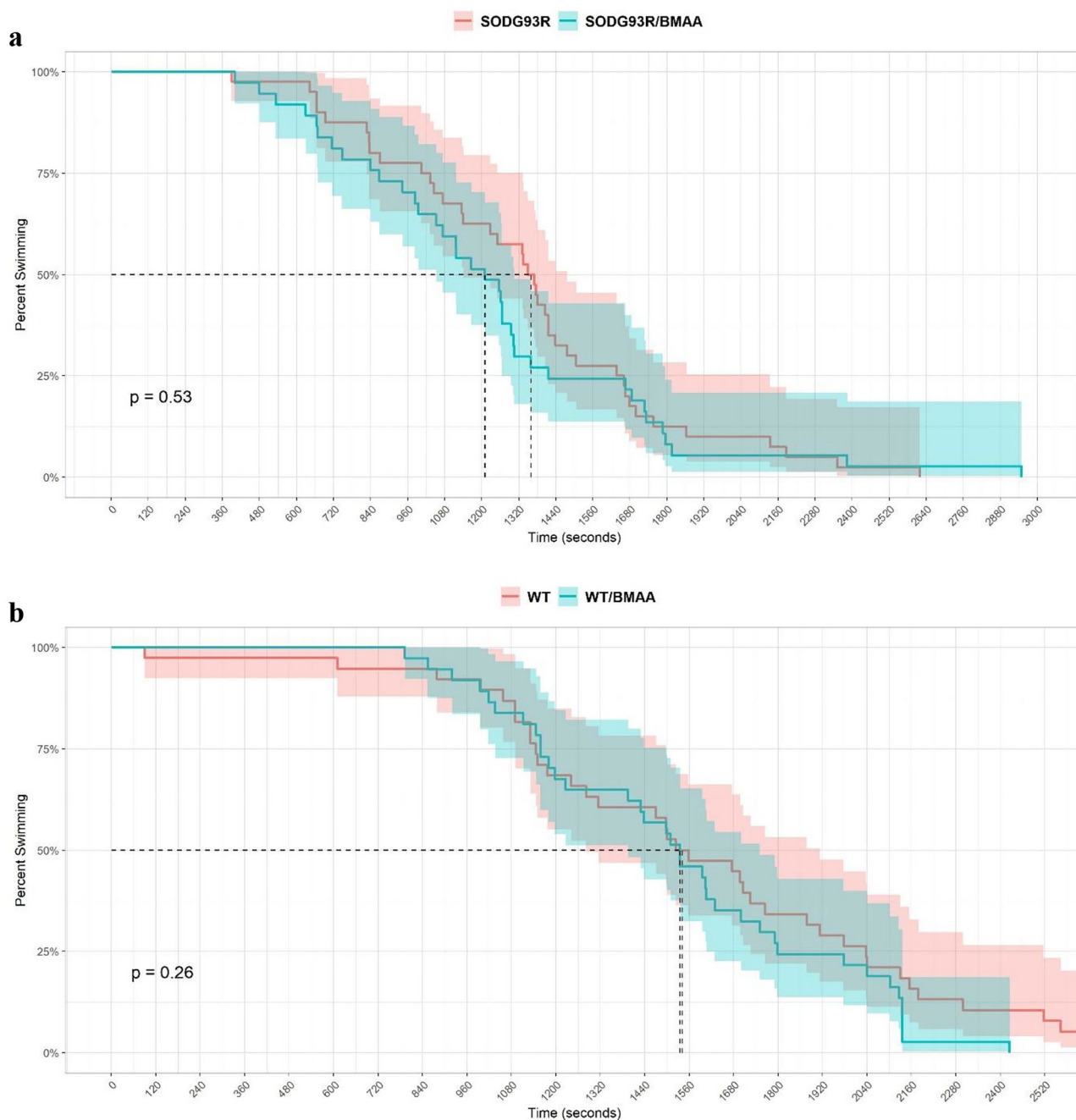


Fig. 4 Swim tunnel assay for long-term BMAA exposures. **a–d** Kaplan–Meier curves represent the amount of time fish were able to swim against a current with increases in flow rate every 120 s. Shaded areas

represent the 95% confidence interval for swim probability at each time point. Dotted lines represent median swim time of individuals in each treatment group

fish than observed in mammals, where BMAA was found to quickly bind to proteins (Xie et al. 2013). The detection of DAB in all of the brain tissue samples was not surprising since this is ubiquitous in the environment; exposure could potentially occur through the commercial fish diet that the fish received. Derivatization with NIFE of the centrifuged brain tissue supernatant was analyzed by MS, indicating the presence of the free enantiomers *L*-BMAA

and *L*-DAB, but not *D*-BMAA or *D*-DAB. Previous chiral analysis of free BMAA in non-human primates and mice found that the majority of BMAA in both the brain and CSF were the *D*-enantiomer even though the animals were fed *L*-BMAA (Metcalf et al. 2017). This suggested that *L*-BMAA is converted to *D*-BMAA within the brain of these mammals with the possibility that an enzyme such as serine racemase recognizes BMAA as a substrate (Baumgart

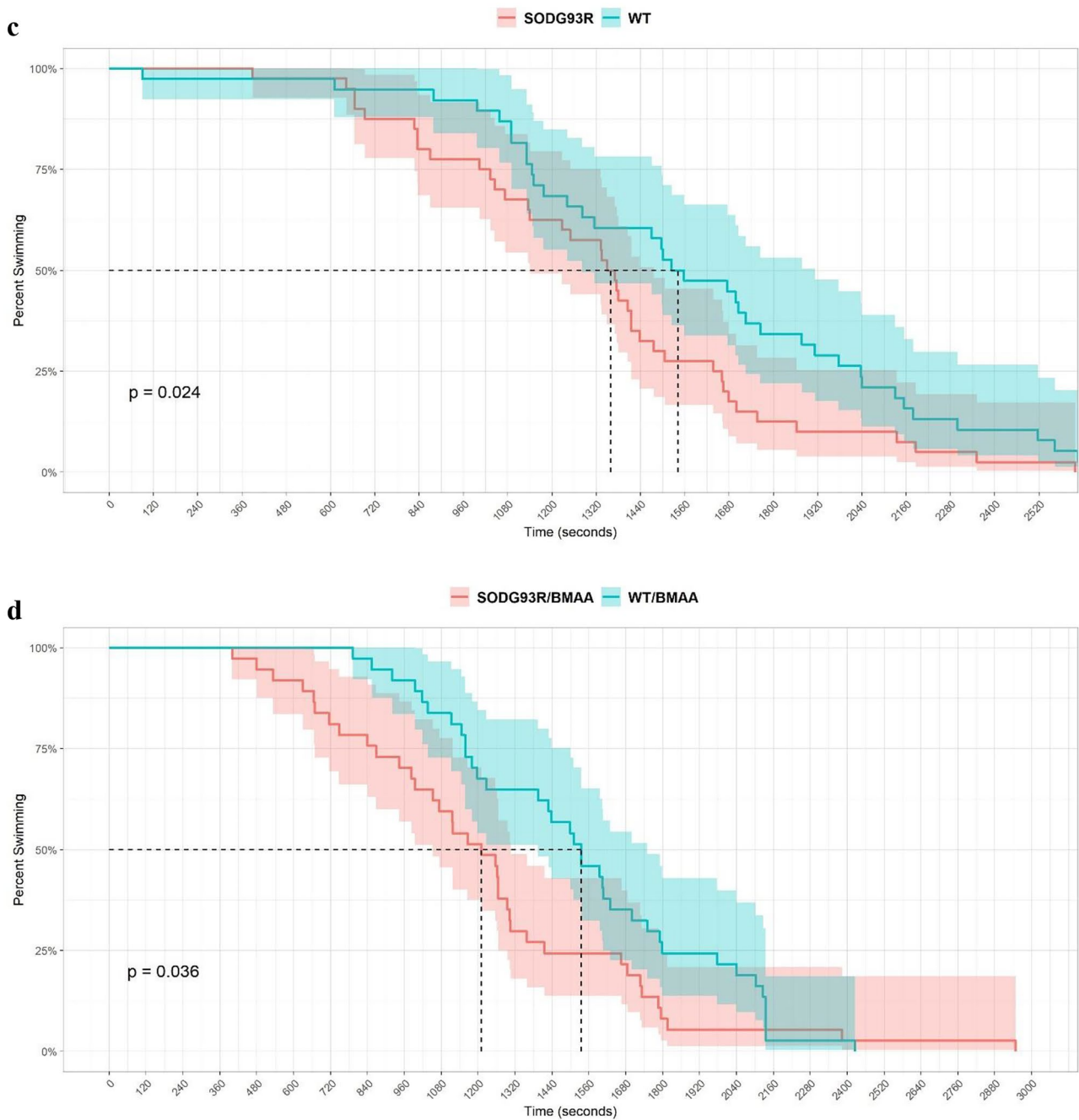


Fig. 4 (continued)

and Rodríguez-Crespo 2008; Dunlop and Neidle 2005). Although the zebrafish genome contains a serine racemase gene (Gene ID: 100,331,498) orthologous to human serine racemase, here again, the comparison with fish brains indicates perhaps different molecular mechanisms within fish compared with what has been observed in mammals.

Analysis of the 100 μ M BMAA diet and 100 mM BMAA stock solution revealed the presence of BMAA in the food, but at values less than anticipated (Table 3). An average of the

TCA and total hydrolyzed BMAA extractions of 12 μ g/mL converts to a concentration of 80 μ M in the liquid diet, 20 μ M less than expected. Similarly, the concentration of BMAA in the stock solution was 12 mg/ml (80 mM) while 100 mM was expected. Previous work suggests that BMAA reconstituted in water is capable of attaching to surfaces and can potentially experience loss while in storage, particularly when mildly acidic conditions are absent (Cohen 2012). Our samples were prepared in water, shipped warm, and stored for about a month

Table 1 MS results of AQC-derivatized brain tissue

AQC derivatized	Free (ng/g tissue)			Protein bound (ng/g tissue)		
	AEG	BMAA	DAB	AEG	BMAA	DAB
Set 1						
WT	ND	ND	46	ND	ND	ND
WT/BMAA	ND	35	51	ND	ND	ND
SOD ^{G93R}	ND	ND	54	ND	ND	ND
SOD ^{G93R} /BMAA	ND	14	24	ND	ND	ND
Set 2						
WT	ND	ND	42			
WT/BMAA	ND	28	41			
SOD ^{G93R}	ND	ND	44			
SOD ^{G93R} /BMAA	ND	28	45			

ND not detected

Table 2 MS results of NIFE-derivatized brain tissue

NIFE derivatized	Free (ng/g tissue)			
	D-BMAA	L-BMAA	D-DAB	L-DAB
Set 1				
WT	ND	ND	ND	231
WT/BMAA	ND	5.8	ND	144
SOD ^{G93R}	ND	ND	ND	159
SOD ^{G93R} /BMAA	ND	NQ	ND	134
Set 2				
WT	ND	ND	ND	79
WT/BMAA	ND	5.8	ND	147
SOD ^{G93R}	ND	ND	ND	167
SOD ^{G93R} /BMAA	ND	5.3	ND	164

ND not detected, NQ not quantifiable

Table 3 MS results of food and tank water

	TCA (µg/mL)	Hyd (µg/mL)
100 mM BMAA stock	11,591	13,416
Liquid diet (100 µM BMAA)	13.5	11.2
Larval food (used to prepare liquid diet)	ND	ND
System water (used to prepare liquid diet)	ND	ND
Adult flake food	ND	ND
Tank water before feeding	0.11	0.14
Tank water 20 min post-feeding	0.03	ND
Blank	ND	ND

ND not detected

at 4 °C prior to analysis, potentially resulting in BMAA loss under these conditions. Nevertheless, the actual food given to the fish over the course of the study was made fresh at least twice per week and stored at 4 °C to minimize loss.

Questions remain concerning the influence of BMAA on disease (Chiu et al. 2011; Beri et al. 2017; Caller et al. 2018; Karamyan and Speth 2008), and as such, many variables have to be considered when interpreting the data including model systems, dosage concentrations, methods of exposure, and trial conditions. Although we have strong experimental evidence that chronic exposure to BMAA causes the neuropathology of ALS in non-human primates (Davis et al. 2020), the taxonomic differences between non-human primates and the zebrafish model are large and the gene-by-environment interactions involved are not yet known. Our work herein with zebrafish and chronic low-concentration BMAA exposure demonstrated no evidence of decreased swim performance, behavioral differences, or any other phenotypes that might be reflective of neurodegenerative disease in these animals. It is possible that the low concentration of BMAA present in the brain tissue of BMAA-exposed fish was insufficient to elicit a phenotypic response, suggesting that additional long-term studies with higher concentrations of BMAA might be needed. It is also possible that adult zebrafish co-evolving with blue-green algae have converged on a BMAA clearance mechanism different from that seen in mammals or in larval zebrafish. Further investigations into these differences could provide fruitful mechanisms of action that could be targeted as protective therapy.

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Author Contribution Ryan Weeks: primary researcher responsible for planning and conducting experiments, along with fish husbandry; assisted with mass spectrometry sample preparation. Sandra Banack: conducted all mass spectrometry experiments and analysis. Shaunacee Howell: aided in conducting behavioral experiments and fish husbandry. Preethi Thunga: responsible for swim tunnel data statistical analysis. Adrian Green: aided in experimental design and behavioral assays, responsible for light/dark assay data analysis. Paul Cox: collaborating PI who advised and directed mass spectrometry experiments. Antonio Planchart: primary PI responsible for project, including mentoring, experimental design, data analysis, and major funding.

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Availability of Data and Materials Contact corresponding author for access to data and materials.

Declarations

Ethical Approval All fish husbandry, anesthesia, and euthanasia were conducted in accordance with NC State Institutional Animal Care and Use Committee (IACUC)-approved protocols.

Competing interests The authors declare no competing interests.

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