Published in final edited form as:

Toxicology. 2019 May 15; 420: 73–84. doi:10.1016/j.tox.2019.04.005.

Exposure to arsenic during embryogenesis impairs olfactory sensory neuron differentiation and function into adulthood

Dana B. Szymkowicz¹, Kaleigh C. Sims¹, Katey L. Schwendinger², Caroline M. Tatnall², Rhonda R. Powell³, Terri F. Bruce³, William C. Bridges⁴, and Lisa J. Bain^{1,2,*}

¹Environmental Toxicology Graduate Program, Clemson University, Clemson, SC, USA

²Department of Biological Sciences, Clemson University, Clemson, SC, USA

³Clemson Light Imaging Facility, Clemson University, Clemson, SC, USA

⁴School of Mathematical and Statistical Sciences, Clemson University, Clemson, SC, USA

Abstract

Arsenic is a contaminant of food and drinking water. Epidemiological studies have reported correlations between arsenic exposure and neurodevelopmental abnormalities, such as reduced sensory functioning, while *in vitro* studies have shown that arsenic reduces neurogenesis and alters stem cell differentiation. The goal of this study was assess whether arsenic exposure during embryogenesis reduced olfactory stem cell function and/or numbers, and if so, whether those changes persist into adulthood. Killifish (*Fundulus heteroclitus*) embryos were exposed to 0, 10, 50 or 200 ppb arsenite (As^{III}) until hatching, and juvenile fish were raised in clean water. At 0, 2, 4, 8, 16, 28 and 40 weeks of age, odorant response tests were performed to assess specific olfactory sensory neuron (OSN) function. Olfactory epithelia were then collected for immunohistochemical analysis of stem cell (Sox2) and proliferating cell numbers (PCNA), as well as the number and expression of ciliated (calretinin) and microvillus OSNs (G_{ai3}) at 0, 4, 16 and 28 weeks. Odorant tests indicated that arsenic exposure during embryogenesis increased the start time of killifish responding to pheromones, and this altered start time persisted to 40 weeks post-exposure. Response to the odorant taurocholic acid (TCA) was also reduced through week 28, while responses to amino acids were not consistently altered. Immunohistochemistry was used to determine whether changes in odorant responses were correlated to altered cell numbers in the olfactory epithelium, using markers of proliferating cells, progenitor cells, and specific OSNs. Comparisons between response to pheromones and PCNA+ cells indicated that, at week 0, both parameters in exposed fish were significantly reduced from the control group. At week 28, all exposure are still significantly different than control fish, but now with higher PCNA expression coupled with reduced pheromone responses. A similar trend was seen in the comparisons between Sox2-expressing progenitor cells and response to pheromones, although Sox2 expression in the 28

*Corresponding author at: Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634 USA .

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Conflicts of interest

The authors declare that there are no conflicts of interest.

week-old fish only recovers back to the level of control fish rather than being significantly higher. Comparisons between calretinin expression (ciliated OSNs) and response to TCA demonstrated that both parameters were reduced in the 200 ppb arsenic-exposed fish in at weeks 4, 16, and 28. Correlations between TCA response and the number of PCNA+ cells revealed that, at 28 weeks of age, all arsenic exposure groups had reductions in response to TCA, but higher PCNA expression, similar to that seen with the pheromones. Few changes in G_{ai3} (microvillus OSNs) were seen. Thus, it appears that embryonic-only exposure to arsenic has long-term reductions in proliferation and differentiation of olfactory sensory neurons, leading to persistent effects in their function.

Keywords

arsenic; olfactory sensory neuron; embryogenesis; killifish; odorant

1. Introduction

Arsenic (As) is a naturally occurring metalloid found throughout the world. Millions of people are exposed to arsenic daily through contaminated drinking water and food sources such as rice. The current drinking water standard set by the World Health Organization is 10 parts per billion (ppb), or 10 $\mu\text{g/L}$. However, some countries have standards set at 50 ppb, and many groundwater sources and private wells exceed these regulations (Mandal and Suzuki, 2002; Naujokas and Anderson, 2013; Ng et al., 2003). The goal of these drinking water standards is to protect against carcinogenic effects, but it is unknown if they are also protective of non-carcinogenic effects of arsenic.

Studies have established a correlation between neurologic deficits and arsenic exposure. Low level arsenic exposure in adults has produced sensory neuropathies and abnormal sensory nerve function, in addition to irritability, depression, and sleep disorders (Mukherjee et al., 2003). Further epidemiological studies link arsenic exposure to decreased intelligence quotients, long-term memory, and neuropsychological changes (Kapaj et al., 2006; Tolins et al., 2014; Yorifuji et al., 2016). Arsenic is known to cross both the placenta and the blood-brain barrier, which poses significant concern for fetuses that are exposed through maternal drinking water (Tolins et al., 2014). Exposure to arsenic through maternal intake produced significant reductions in verbal and full-scale IQ in female children up to five years old who were exposed both prenatally and during early life (Hamadani et al., 2011). Negative associations were also seen between urinary arsenic (<50 ppb) and visual-spatial tests, picture recognition, and problem-solving tests in children 6–8 years of age (Rosado et al., 2007). Similar neurobehavioral changes and memory deficits have been seen in prenatal exposures in rodents. For example, significant delays in righting reflexes, cliff aversions, and negative geotaxis were seen in female rats exposed to 50 and 100 ppb arsenic in maternal drinking water (Gumilar et al., 2015).

Although the mechanisms as to how arsenic impairs neuronal development are not fully known, many studies have reported that arsenic exposure reduces the proliferation, differentiation, and function of multiple stem cell types. For example, arsenic exposures as low as 7.5 ppb inhibited the differentiation of mouse embryonic stem cells into sensory

neurons by reducing the expression of NeuroD, neurogenin 1, and neurogenin 2 transcription factors (Hong and Bain, 2012; McCoy et al., 2015). Similarly, human neural stem cells exposed to arsenite had reduced differentiation (Baek et al., 2012; Ivanov and Hei, 2013). *In vivo*, adult rats exposed to arsenic for two months had reduced numbers of neural progenitor cells (Liu et al., 2012), while mice exposed throughout the perinatal period had decreased formation of new neurons (Tyler and Allan, 2013) due to delayed differentiation of embryonic neural progenitor cells (Tyler et al., 2017).

Proper number and function of neural stem cells is essential for the development and maintenance of the olfactory system, and altered sensory responses have been correlated with reduced neuronal differentiation (Cronican et al., 2013; Luo et al., 2013; Zhang et al., 2014). Neurons involved in olfaction are produced by neural stem cells both during development and throughout adulthood, making the olfactory system an ideal model to study both embryonic and adult neurogenesis (Lindsey et al., 2014). The olfactory system is highly conserved across vertebrates. In fish, the olfactory system is crucial for various sensory behaviors needed for reproduction, feeding, socialization, and predator avoidance. Olfaction occurs through activation of G-protein coupled receptors (GPCRs) on olfactory sensory neurons (OSNs), which are passed through the olfactory nerve and into the olfactory bulb and sent to the telencephalon for processing (Bazáes et al., 2013; Hino et al., 2009). The olfactory epithelium has several cell types that are specific to certain odorants: ciliated OSNs respond to bile salts and steroids, microvillus OSNs respond to amino acids and food cues, and in fish, crypt OSNs are thought to respond to pheromones (Kaupp, 2010). If stem cell populations needed for OSN regeneration are reduced or impaired, neurogenesis could be altered and ultimately lead to sensory dysfunction.

Killifish (*Fundulus heteroclitus*) are a commonly used animal model for developmental studies (Burnett et al., 2007). They produce large, transparent eggs that are fully developed in 14–21 days in laboratory settings. Compared to rodent models, they produce a large number of offspring, and can offer greater statistical power. Killifish metabolize arsenic also in a manner more similar to humans (Sims et al., 2019) compared to rodent models, which have differences in arsenic methylation efficiency (Rehman and Naranmandura, 2012). Previous studies have exposed killifish to 0–800 ppb arsenic during embryogenesis and reported reductions in their response to a food stimulus (Szymkowicz et al., 2017).

Whether arsenic can impair neurogenesis, and therefore alter sensory outputs and behavior, is not known. In the current study, we exposed killifish embryos to environmentally relevant concentrations of arsenic during the embryonic period and raised the hatchlings in clean water for up to 40 weeks. The goal was to examine whether arsenic reduced neuronal stem cell populations or their differentiation, and if so, whether these changes persisted into adulthood.

2. Methods

2.1. Killifish (*Fundulus heteroclitus*) collection and sodium arsenite exposure

Adult male and female killifish were collected from the Baruch Institute for Marine and Coastal Ecology at the North Inlet National Estuarine Research Reserve (NERR) in

Georgetown, SC and maintained in the Aquatic Animal Research Laboratory at Clemson University under IACUC protocol 2016–038. Adult killifish were housed in 18 ppt salt water (Coralife; Rhinelander, WI) on a 16:8 light/dark cycle at 26°C. Fish were fed zebrafish pellet food (Zeigler Brothers; Gardners, PA) twice a day prior to egg collection and fertilization.

To observe early olfactory injury after arsenic exposure during embryogenesis, eggs and milt were collected from male and female adult fish, and mixed together for fertilization. Approximately 2,200 fertilized eggs were randomly separated into 24 Petri dishes. Embryos were exposed to 0, 10, 50 or 200 ppb sodium arsenite (n=6 dishes per group; 40 eggs per dish) (Fisher Scientific; Fair Lawn, NJ). Killifish were removed from the arsenite exposure within 24 hours of hatching and placed into 3.7 L zebrafish tanks with arsenite-free 18 ppt salt water (Coralife; Rhinelander, WI) for grow out. Juvenile fish were fed brine shrimp twice a day. Odorant responses tests were conducted, and killifish were randomly sampled and euthanized at 0, 2, and 4 weeks post-hatch for olfactory epithelia collection.

To observe long-term effects of arsenic exposure during embryogenesis, and to avoid overcrowding, a second set of fertilized eggs were collected and randomly dispersed into 28 Petri dishes. As with the early time point study, embryos were exposed to 0, 10, 50 or 200 ppb sodium arsenite (n=7 dishes per exposure group). Within 24 hours of hatching, juvenile fish were removed from the Petri dishes and placed into 10 gallon tanks of arsenite-free 18 ppt salt water for grow out. Juvenile killifish were maintained on a brine shrimp diet until 4 weeks of age then gradually switched to Zeigler adult zebrafish diet (Gardners, PA). Zeigler food was analyzed for inorganic arsenic content and non-detectable levels were reported (data not shown). Odorant response tests were conducted, and killifish were sampled at 8, 16, 28 and 40 weeks of age for olfactory epithelia collection.

2.2. Odorant behavioral tests

To look for initial injury to olfactory sensory neurons (OSNs), odorant response tests were performed. At week 0, hatchlings were removed from the exposure Petri dishes and placed into 3.7 L zebrafish tanks. After 70% of the embryos hatched, the test was initiated. Behavior was initially recorded for 20 seconds using a Canon video camera to capture “normal” behavior. Then, 1 mL of a 250 mM taurocholic acid (TCA) solution was added into the top of the water column in each tank to induce a socialization response, and fish behavior was video recorded for an additional 60 seconds. Two additional behavior tests were conducted over the next 24 hours using 1 mL of an amino acid mixture (250 mM each of L-methionine, L-histidine, L-cysteine, L-valine, L-alanine) to induce a feeding response, and with 0.4 mL of killifish pheromone extract (1.5g female egg sacs homogenized in 30 mL phosphate-buffered saline) to induce a mating response. At weeks 2 and 4, behavior tests were performed once every 8–10 hours in the 24-hour time period before euthanasia. Videos were analyzed for time to start, which was determined as the time when 10% of the total fish started responding to the odorant, and percent response of killifish.

To look for long-term changes in olfactory function, the same odorant response tests were performed at 8, 16, 28 and 40 weeks of age. Three weeks prior to each sampling time point, killifish were exposed to one of the three different odorants each week. Behavior tests for each odorant were repeated once more within the week, as described above. Due to the

larger tank size, 4 mL of the 250 mM amino acid and TCA mixtures were used, and 1 mL of the pheromone extract was used. Killifish behavior was recorded for 20 seconds before addition of each odorant to observe “normal” behavior. After the odorant addition, behavior was video recorded for an additional 60 seconds for amino acids and pheromones, and 120 seconds for TCA. Videos were analyzed for time to start and percent response of killifish.

2.3. Western blotting for antibody validation

Control adult killifish were euthanized using buffered MS-222 and samples of olfactory epithelia were collected for protein extraction. Immunoblotting was used to determine whether antibodies for PCNA, Sox2, calretinin, and G_{ai3} cross-reacted in killifish olfactory epithelial tissue. Tissues were homogenized in RIPA buffer with protease inhibitors and were centrifuged at 10,000 rpm for 15 minutes at 4 °C. Protein concentrations were determined using the Bradford assay (BioRad; Hercules, CA) and 30 µg of protein was electrophoresed onto SDS polyacrylamide gels. Following transfer to nitrocellulose, primary antibodies for PCNA (#sc-56; Santa Cruz Biotechnology, Dallas, TX), Sox2 (ab97959; Abcam), calretinin (ab702; Abcam), and G_{ai3} (sc365422; Santa Cruz Biotechnology, Dallas, TX) were each used at 1:1000 dilution. AP-labeled anti-mouse and anti-rabbit secondary antibodies were used at 1:2500 dilution. Blots were developed using NitroBT and BCIP and imaged with a BioRad gel imager.

2.4. Immunohistochemical analysis of olfactory epithelia

To assess changes in numbers and localization of cells in the olfactory epithelia, one fish per tank was euthanized in 1 g/L buffered MS-222 at each of our time points, weeks 0, 4, 16, and 28, and olfactory epithelia were collected. Tissue was fixed overnight in 10% neutral-buffered formalin, then processed and embedded in paraffin. Olfactory epithelia were cut into 5 µm sections. Antibodies for proliferating cells (PCNA; sc-56), neural stem cells (Sox2; ab97959), ciliated cells (calretinin; ab702), and microvillus cells (G_{ai3} ; sc-365422) were used to quantify each cell type. Antigen retrieval was performed using sodium citrate pH6 buffer (PCNA, Sox2, G_{ai3}) or Tris-EDTA pH9 buffer (calretinin). Samples were blocked overnight at 4 °C in 5% BSA, 10% goat serum, and 0.1% Tween 20. Primary antibodies were used at 1:100 dilution and incubated overnight at 4°C. Alexa Fluor 488 anti-mouse and Alexa Fluor 532 anti-rabbit secondary antibodies were used at 1:200 dilution for 1hr at room temperature. All samples were counterstained with DAPI. For each antibody tested, one fish per tank at each time point was examined.

Samples stained with PCNA or Sox2 were imaged using a Leica SPE confocal microscope, equipped with traditional PMT detectors and a 63X objective. For PCNA, Sox2, and DAPI, excitation wavelengths were 488, 532, and 406 nm, respectively, and emission wavelengths were 500–560, 535–600, and 430–480 nm, respectively. Samples stained with calretinin and G_{ai3} were imaged using a Leica SP8X confocal microscope, equipped with HyD detectors, time gating, a 63X objective (N.A= 1.4) with 1.5 zoom, and a tunable laser. An excitation wavelength of 532 nm with emission wavelengths of 540–595 nm were used for calretinin, and excitation wavelength of 495 nm with emission wavelengths of 498–560 nm was used for G_{ai3} . Imaging conditions (laser power and gain) were held consistent for all samples, exposure groups, and time points when examining PCNA, Sox2, and calretinin expression.

While examining G_{ai3} intensities, changes in laser power were needed at different weeks in order to obtain appropriate images. Images were analyzed using ImageJ (NIH). For PCNA and Sox2, the number of cells expressing each antigen was counted ($n = 4-6$ tanks; 1 fish per tank). For calretinin and G_{ai3} , average integrated density was determined ($n = 4-6$ tanks; 1 fish per tank). Briefly, the olfactory region was selected using the hand tool in ImageJ, threshold was set, and regions of interest (ROIs) were generated. Integrated density (sum of values of the pixels per given area) was quantified then averaged across replicates. At each time point, calretinin intensities were normalized to week 0 controls to allow for comparisons across weeks. Due to differences in laser power, G_{ai3} intensities were not normalized to week 0 for comparison across time points.

2.5. Statistical analysis

The dependent variables were averaged across the technical replicates. Differences in start time, duration and percent response were averaged across replicates ($n=6-7$ tanks) for each exposure group. Number of PCNA and Sox2 cells were also averaged across replicates ($n=4-6$ tanks; 1 fish per tank) for each exposure group at each time point. Integrated density of calretinin and G_{ai3} were converted to fold change of week 0 control and averaged across replicates ($n=4-6$ tanks; 1 fish per tank). All dependent variable averages had equal variances and normal distributions, so parametric statistical tests were used.

Overall differences among means of the individual dependent variables across the arsenic exposure groups were evaluated using an analysis of variance (ANOVA). Specific differences between the means of the exposure groups were evaluated using Tukey's post-hoc test. The ANOVA and Tukey's analyses were performed per time point for dependent variables measured over time. Overall differences in the means of the combined dependent variables of percent response and cell counts (PCNA, Sox2) across the arsenic exposure groups were evaluated using a multivariate analysis of variance (MANOVA). Specific differences among the combined means across exposures were further evaluated with contrasts. The MANOVA and contrasts were performed per time point. Differences in the individual dependent variables (ANOVA and Tukey's) and the combined dependent variable means (MANOVA and contrasts) were used together to determine if relationships existed between the percent response and cell counts across the exposure groups. All statistical calculations were performed using JMP Pro 12 and $p < 0.05$ was considered evidence of statistical significance.

3. Results

3.1. Arsenic exposure during embryogenesis reduces behavioral responses to pheromones

Odorant response tests were used to determine if embryonic arsenic exposure altered the development, differentiation, or function of olfactory sensory neurons, and assess whether these changes persisted into adulthood. Killifish ovarian extracts were used as a pheromone odorant to activate crypt OSNs, and all behavioral tests were conducted on a per-tank basis. At week 0, there was a significant dose-dependent increase in start time, in which exposed killifish take 1.7- to 1.9-fold longer to respond (Fig. 1A; $p = 0.001-0.04$). At week 2, only

the 200 ppb exposed fish take significantly longer to respond to the pheromones ($p = 0.01$), and by week 4, there are no longer changes in start time (Fig. 1A). However, as the fish age, the 10 and 50ppb groups have a significant 1.5- to 1.6-fold reduction in start time by 8 weeks of age (Fig. 1A; $p = 0.0003$ – 0.03). At weeks 16 and 28, all three exposure groups have significantly greater start times ($p = 0.003$ – 0.04). By 40 weeks of age, we can visually determine the sex of the fish. There were no differences in the sex ratios between exposure groups (control group was an average of $54.7 \pm 27\%$ males; 10 ppb group was $40.1 \pm 13.4\%$ males; 50 ppb group was $50 \pm 10.8\%$ males; 200 ppb group was $41.7 \pm 10.1\%$ males). The arsenic-exposed 40 week-old fish still take a longer period of time to start their response to pheromones than the control fish do (Fig. 1A). The percentage of fish responding to pheromones is concordant to the delayed start times, in that at week 0, percent response is also significantly reduced by 1.7- to 1.9-fold in exposed killifish (Fig. 1B) and this reduction remains for the 200 ppb exposure group until week 4. At weeks 16 and 28, the percentage of fish responding is significantly reduced in most of the exposure groups by 1.3- to 1.6-fold (Fig. 1B; $p = 0.001$ – 0.05).

3.2. Arsenic exposure during embryogenesis impairs socialization responses following a TCA stimulus

Taurocholic acid (TCA) is a bile salt that is commonly used to initiate socialization responses in fish, and exposure to this odorant typically activates ciliated OSNs. At week 0, there were no significant differences in start time or percent response. At week 2, time to start responding was significantly delayed by 1.5-fold in 200 ppb fish ($p = 0.04$), while by week 4, start time was significantly delayed in 50 and 200 ppb fish by 1.7-fold (Fig. 2A; $p = 0.011$ – 0.016). While trends exist for delayed start time in the remaining weeks, only week 28 fish were statistically different from controls (Fig. 2A; $p = 0.007$ – 0.03). Similarly, the percentage of fish responding to TCA was significantly impaired, with 1.7- to 2.2-fold less fish responding at week 2, and 1.6- to 1.7-fold less fish responding at week 28 (Fig. 2B; $p < 0.045$). By week 40, there continued to be a dose-dependent trend in percent response, with a 1.5-fold reduction in 200 ppb fish (Fig. 2B).

3.3. Arsenic exposure during embryogenesis reduces behavioral responses to amino acids only early in life

To assess activation of microvillus OSNs, an amino acid mixture was administered to the water column of each tank to induce a feeding response. At week 0, there were no differences in start time in any exposure group. At weeks 2 and 4, time to start was significantly increased by 131–302% in 50 and 200ppb fish (Fig. 3A; $p = 0.012$ – 0.021). After these earlier changes, there is either no or minor changes in start time as the fish age. For example, the 28 week fish at 50 ppb show a statistically significant reduction in response and an increase in time to start, this is driven by two tanks of fish with very low response rate. In a similar fashion, percentage of fish responding to amino acids is impaired early on, with reductions in fish exposed to 200 ppb arsenic at weeks 0 and 2 (Fig. 3B; $p = 0.012$ – 0.017), but these differences disappear as the fish age.

3.4. Arsenic exposure during embryogenesis alters PCNA and Sox2 expression in killifish

Immunohistochemistry was used to assess stem cell populations in the olfactory epithelium in a subset of ages, at 0, 4, 16 and 28 weeks, to determine if embryonic arsenic exposure alters the number of proliferating cells (PCNA) or the number of neural stem cells (Sox2) both immediately after exposure (Fig. 4) and later in life (Fig. 5; Supp. Fig. 1,2). At week 0, PCNA expression trends downwards and Sox2 expression is reduced by 35% in fish exposed to 50 ppb arsenic (Fig. 6A, B; $p = 0.04$). At week 4, the reduced PCNA and Sox2 expression trends still exist (Fig. 6A, B). However, at week 16, PCNA expression is increased by 65% in 200 ppb fish ($p = 0.04$) and Sox2 expression remains suppressed by 23–34% (Fig. 6A, B; $p = 0.02$ – 0.05). By week 28, PCNA expression remains elevated by 43–68% in 50 and 200 ppb fish ($p = 0.001$ – 0.04). Sox2 expression is no longer affected by arsenic exposure at week 28 (Fig. 6B).

3.5. Arsenic exposure during embryogenesis alters calretinin expression in ciliated OSNs

Immunohistochemistry was also used to determine if embryonic arsenic exposure affected the presence of specific olfactory cell types in the epithelium. Multiple antibodies were tried to assess the presence of crypt cells, but none reacted with killifish epitopes. Therefore, expression of calretinin (ciliated cells) and G_{ai3} (microvillus cells) were analyzed (Fig. 4–5; Supp. Fig. 1–2). In order to compare changes in expression as the fish aged, calretinin expression was normalized to week 0 controls, while G_{ai3} expression was compared to the control at each time point due to variable microscope laser settings. At week 0, there are few differences between control and exposed fish when examining calretinin and G_{ai3} expression (Supp. Fig. 3). At weeks 4, 16, and 28, there is a dose-dependent decreasing trend in calretinin expression, while the expression of G_{ai3} is only altered at week 16 (Supp. Fig. 3).

3.6. Relationship between IHC and odorant responses

Following analysis of both behavioral responses and olfactory epithelium protein expression by IHC, we wanted to look for relationships between specific cell types and their functions. Relationships between PCNA expression (proliferation) and pheromone response were first examined. At week 0, all exposure groups are significantly different from the control group (based on MANOVA and contrasts), driven by reductions in both percent response to pheromones and PCNA expression (Fig. 7A; $p = 0.006$ – 0.03). At week 4, the 200ppb group is still different than the control due to lower levels of PCNA (Fig. 7B; $p = 0.001$). By week 16, however, PCNA expression increases in the 200ppb group even though response to pheromones is still lower than the controls (Fig. 7C). By week 28, all exposed groups are significantly different than the controls, which have higher expression of PCNA coupled with reductions in response to pheromones (Fig. 7D; $p < 0.0001$). When comparing the number of neural stem cells, as measured by Sox2 expression, with the response to pheromones, the 50 and 200ppb groups are significantly different than controls at week 0 due to both lower Sox2 levels and reduced percent response (Fig. 7E; $p = 0.001$ – 0.005). At week 4, there are no significant differences between control and exposure groups, although there remains a decreasing trend in percent response (Fig. 7F). By week 16, all three exposure groups are still significantly different than the control due to reduced Sox2 levels

and reduced pheromone responses (Fig. 7G; $p = 0.001$ – 0.03). At week 28 there seems to be a recovery in Sox2 expression but reductions in percent response to pheromones persist (Fig. 7H; $p < 0.001$).

Next, we performed the MANOVA and contrasts to compare the response to TCA with the number of proliferating and differentiating cells (Fig. 8). At week 0, lower PCNA levels and response to TCA drive the differences between the 50 and 200 ppb groups and the control group (Fig. 8A; $p = 0.03$ – 0.05). At week 4, the 10 and 200 ppb groups are significantly different than the controls mostly due to reduced PCNA expression (Fig. 8B; $p = 0.001$ – 0.05). By week 16, however, PCNA levels are starting to increase in the exposed groups compared to the control while the TCA response still remains dampened (Fig. 8C; $p = 0.002$). By week 28, all exposure groups have higher levels of PCNA and reduced percent response to TCA as compared to the controls (Fig. 8D; $p = 0.001$ – 0.003).

When comparing Sox2 expression to percent response to TCA, week 0 fish embryonically exposed to the two higher levels of arsenic have both reduced Sox2 expression and reduced TCA response (Fig. 8E; $p = 0.04$ – 0.05). While changes were modest at week 4 (Fig. 8F), by week 16, all exposure groups are significantly different than the control group due to both lower Sox2-expressing cells and reduced response to TCA (Fig. 8G; $p = 0.001$ – 0.02). However, by week 28, Sox2 expression has increased in the exposed groups, and the significant differences are due to reductions in response to TCA (Fig. 8H; $p = 0.004$ – 0.04).

Finally, we compared the response to TCA with the expression of calretinin, a marker for ciliated OSNs that respond to bile salts (Fig. 9). At week 0, the 50 ppb group is significantly different than the control group due to elevated levels of calretinin (Fig. 9A; $p = 0.012$). At week 4, a dose-dependent trend is seen with reductions in both percent response to TCA and calretinin expression, with the 200ppb group being significantly different (Fig. 9B; $p = 0.023$). At week 16, the 50 and 200 ppb groups are significantly different from the control due to reductions in response to TCA (Fig. 9C; $p = 0.008$ – 0.04), while at week 28, all three groups are significantly different then the control group (Fig. 9D; $p = 0.002$ – 0.02).

Relationships between IHC markers and start time for each odorant were very similar to the relationships with percent response (data not shown). MANOVA and contrasts were also performed for response to amino acids and the numbers of PCNA or Sox2 positive cells (Supp. Fig. 4). At week 0, all three groups are different from the control due to PCNA levels (Supp. Fig. 4A; $p = 0.02$ – 0.05), which in later weeks shifts to increased PCNA expression without much change in the response to amino acids. These differences remain at week 28 (Supp. Fig. 4D; $p = 0.001$ – 0.03). For Sox2, any significant differences are mostly due to changes in Sox2 levels (Supp. Fig. 4E–H). MANOVA and contrasts were also done to compare response to amino acids and G_{ai3} expression, the marker for microvillus OSNs, but few changes were seen (data not shown).

4. Discussion

The results from this study indicate that arsenic exposure during embryogenesis to environmentally-relevant levels of arsenic causes a delay in cellular proliferation and

reduced differentiation of neural stem cells in the olfactory epithelium. The impairment in turn produced reductions in ciliated OSNs, and responses to pheromones, that persisted into adulthood, long after the exposure was removed.

4.1. Arsenic exposure during embryogenesis delays proliferation and differentiation of neural stem cells in the olfactory epithelium

During early development of the olfactory system, OSNs are produced through expression of transcription factors such as Foxg1, Neurogenin1, NeuroD4, and Cxcr4b, which is followed by cellular migration and proliferation within the olfactory placode region (Miyasaka et al., 2013). Basal stem cells located in the epithelium initially differentiate into neuroblasts, which become further specified into microvillus, ciliated, or crypt OSNs for functional tasks of responding to specific odorants. Throughout the lifetime of both fish and mammals, adult neurogenesis is ongoing in the olfactory epithelium (Lindsey et al., 2014; Cheetham et al., 2016). The repopulation from the stem cells happens over a period of 10–14 days. For example, recovery of damaged OSNs has been seen in zebrafish two weeks after chemical lesioning and exposures to zinc sulfate (White et al., 2015; Hentig and Byrd-Jacobs, 2016). The inability of stem cells to appropriately proliferate and differentiate throughout adulthood could lead to sensory neuron dysfunction.

Thus, the goal of this study was to determine whether an embryonic exposure to arsenic would permanently reduce either the number and/or functioning of olfactory epithelial cells. Our results show decreased numbers of proliferating cells, as measured by PCNA, along with reductions in neuroblast populations, measured by Sox2, immediately after hatching. This indicates that arsenic can impair sensory neuron development during embryogenesis. While these initial changes exist, by week 16 of recovery in clean water, there is a dramatic increase in stem cell proliferation that continues through week 28. At week 16, the proliferation of stem cells has not quite translated into the production of neuroblasts, as their numbers are still reduced. However, at week 28 of recovery, the numbers of neuroblasts (Sox2 expression) has increased in the olfactory epithelium. These results suggest that repopulation of the olfactory epithelium can eventually take place following embryonic arsenic exposure, although it does take between four and six months.

The reductions in sensory stem cell differentiation after early-life arsenic exposure are concordant with altered sensory responses seen in epidemiological studies. For example, correlations were seen between urinary arsenic levels and reduced taste and smell abilities in children (Calderón et al., 2001; Rosado et al., 2007; Rodríguez-Barranco et al., 2016; Tsuji et al., 2015). *In vivo* studies in animals have shown similar effects on neurosensory function, where *in utero* exposures to arsenic altered auditory responses and reduced sensorimotor reflexes (Xi et al., 2009; Gumilar et al., 2015). Similarly, epidemiological studies report that adult exposure to arsenic is correlated with sensory neuropathy in the extremities, reduced visuospatial skills, and reduced touch and vibration sensations (Mukherjee et al., 2003; Hafeman et al., 2005; O'Bryant et al., 2011). *In vitro* studies also show that low-level arsenic exposure to mouse embryonic stem cells can specifically impair sensory neuron differentiation (Hong et al., 2012; McCoy et al., 2015). Based on epidemiological, *in vivo*,

and *in vitro* studies, it is clear that sensory neural progenitor cells are targets of arsenic toxicity that lead to neurobehavioral and neurodevelopmental deficits.

4.2. Arsenic exposure during embryogenesis reduces response to pheromones and alters levels of ciliated OSNs

After observing changes in stem cell division in the olfactory epithelium, we also investigated whether they were able to differentiate into functional OSNs. There are three main types of OSNs in fish, each of which are specific to particular odorants. Ciliated OSNs respond to bile salts and steroids, microvillus OSNs respond to amino acids and food cues, and crypt OSNs are thought to respond to pheromones (Kaupp, 2010). Therefore, pheromones, the bile salt taurocholic acid (TCA), and various mixtures of amino acids have been used to assess the impacts of contaminant exposure on the olfactory epithelium. For example, after brief exposures to cadmium or zinc, zebrafish have decreased responses to TCA and L-cysteine (Heffern et al., 2018; Matz and Krone, 2007).

Previous studies using killifish have reported a reduced response to a food stimulus following arsenic exposure during embryogenesis (Szymkowicz et al., 2017). In order to remove the visual stimulus and to target the function of all three types of OSNs, we administered solutions of female adult pheromones, TCA, and an amino acid mixture to activate crypt, ciliated, and microvillus OSNs, respectively. Immediately after hatching, the time it took to start responding to a pheromone odorant was significantly longer in the embryonically-exposed fish compared to control fish. Similar trends were seen with TCA and amino acids, but not to the same extent. By 4 weeks post-exposure, significant delays in start time were seen in response to TCA and amino acids in the exposed fish. Start time in response to pheromones remained delayed throughout the entire 40 weeks of the study in fish exposed to as low as 10ppb arsenic, indicating long-term impairments in crypts OSNs with little recovery. In contrast, differences in start time for TCA ceased after week 28, and ended by week 8 for amino acids, suggesting a recovery of the ciliated and microvillus cells.

As with the behavioral findings, several studies have also found that chemical exposures can directly target specific OSN subtypes. For example, salmon exposed to cadmium had decreased response to L-cysteine that persisted even after a 16-day depuration period, which correlated to severe reduction in the expression of adenylate cyclase III, a signal transduction protein found only in ciliated OSNs (Williams et al., 2016). Copper exposures to salmon have produced similar changes in expression of olfactory sensory genes, specifically those present in ciliated OSNs (Wang et al., 2013). Copper and nickel exposures in zebrafish appear to affect both ciliated and microvillus OSNs, but not crypt cell populations (Lazzari et al., 2017; Lazzari et al., 2018).

In killifish embryonically exposed to arsenic, expression of calretinin, the marker for ciliated cells, and expression of $G_{\alpha 13}$, the microvillus cell marker, were not altered at hatching. However, as the fish aged, there were reductions in calretinin expression in the embryonically-exposed fish. This is likely because neural stem cells must first proliferate, differentiate into neuroblasts, and then further differentiate into ciliated OSNs. At week 28, all groups are expressing calretinin at 3- to 4 times higher levels than at week 0, yet calretinin expression is still reduced. When correlating responses to TCA with calretinin

expression, there are significant differences between control and exposed groups driven by both reduced responses and reduced calretinin expression throughout the six month recovery period. Comparisons between amino acids responses and PCNA, Sox2, and G_{ai3} did not have strong trends, which suggests that arsenic impairs ciliated OSN differentiation more so than microvillus OSNs. While we could assess responses to pheromones and show that arsenic exposure during embryogenesis impairs this response out to 28 weeks of age, crypt OSN numbers could not be assessed due to a lack of specific antibodies that reacted with killifish epitopes. With that, there are conflicting results as to the true function and activation of crypt cells. One study found that a small population of crypt-like immature cells in juvenile rainbow trout that responded to amino acids and bile salts. However, mature crypt cells in adult rainbow trout responded strongly to the gonadal extracts from the opposite sex (Bazáes and Schmachtenberg, 2012). Thus, it appears that arsenic is one of the first chemicals found to impair pheromone responses, although it is unknown whether this is truly due to a reduction in the number of crypt cells.

In summary, arsenic exposure during embryogenesis alters proliferation and differentiation of stem cells in the olfactory epithelium that persists through at least 28 weeks post-exposure. While the numbers of PCNA+ and Sox2+ cells levels seem to be compensating and catching up by week 28, the response to pheromones nor ciliated OSN levels never catches up to the controls. Overall, these results suggest that stem cells in the olfactory epithelium are a potential target of arsenic toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank John Smink for his assistance with killifish husbandry and the Clemson University Histology Core facility. The research was supported by National Institutes of Health (R03ES023930). The immunohistochemical analyses were conducted in the Clemson Light Imaging Facility (CLIF) using instruments funded by the National Institutes of Health (P20GM109094 and P20RR021949), and the National Science Foundation (MRI 1126407).

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Highlights

- Embryonic-only arsenic exposure impairs response to pheromones and taurocholic acid
- Olfactory stem cell numbers are changed even 6 months post-exposure
- Impaired TCA response correlates to reduced ciliated olfactory sensory neurons (OSNs)
- Changes in proliferating and differentiating OSNs correlate with pheromone response

Fig. 1.

Exposure to arsenic during embryogenesis increases start time and reduces killifish response to pheromones. At each time point, 1 mL of adult killifish pheromone extract was added to the top of the water column, and fish response was recorded for 60 seconds. Start time (time for 10% of fish in tank to begin responding) and percentage of fish responding were determined for each exposure ($n = 6-7$ tanks). Data is presented as start time for weeks 0–40 (A) and percent response for weeks 0–40 (B). Statistical differences between control and exposure groups at each time point were determined using one-way ANOVA and Tukey's post-hoc test (*; $p < 0.05$).

Fig. 2.

Changes in start time and response to taurocholic acid (TCA) following an exposure to arsenic during embryogenesis. At each time point, 4mL of 250mM TCA was added to the top of the water column, and killifish behavior was recorded for 120 seconds. Start time and percent response were determined for each exposure ($n = 6-7$ tanks). Data is presented as start time for weeks 0–40 (A) and percent response for weeks 0–40 (B). Statistical differences between control and exposure groups at each time point were determined using one-way ANOVA and Tukey's post-hoc test (*; $p < 0.05$).

Fig. 3.

Changes in start time and response to amino acids following an exposure to arsenic during embryogenesis. At each time point, 4mL of a 250mM amino acid mixture was added to the top of the water column, and killifish behavior was recorded for 60 seconds. Start time and percent response were determined for each exposure ($n = 6-7$ tanks). Data is presented as start time for weeks 0–40 (A) and percent response for weeks 0–40 (B). Statistical differences between control and exposure groups at each time point were determined using one-way ANOVA and Tukey's post-hoc test (*; $p < 0.05$).

Fig. 4.

Representative confocal images of week 0 immunohistochemistry of olfactory epithelia. One fish per tank was euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5 μm . Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and $G_{\alpha i3}$ (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, $G_{\alpha i3}$). Scale bar = 20 μm .

Fig. 5.

Representative confocal images of week 28 immunohistochemistry of olfactory epithelia. One fish per tank were euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5 μm . Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and $G_{\alpha i3}$ (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, $G_{\alpha i3}$). Scale bar = 20 μm .

Fig. 6.

PCNA and Sox2 expression in the olfactory epithelium after an exposure to arsenic during embryogenesis. Following immunohistochemical staining, the number of cells expressing PCNA (A) or Sox2 (B) were counted and averaged across exposures ($n = 4-6$ tanks; 1 fish per tank) at week 0, 4, 16, and 28. Statistical differences between control and exposure groups at each time point were determined using one-way ANOVA and Tukey's post-hoc test (*; $p < 0.05$).

Fig. 7.

Correlations between PCNA/Sox2 and percent response to pheromones. Average number of PCNA-expressing cells for each exposure ($n = 4-6$ tanks; 1 fish per tank) at each time point was plotted against average percent (%) response for pheromone behavior tests for each exposure ($n = 6-7$ tanks) for weeks 0–28 (A–D). Average number of Sox2-expressing cells for each exposure ($n = 4-6$ tanks; 1 fish per tank) at each time point was plotted against average percent (%) response for pheromone behavior tests for each exposure ($n = 6-7$

tanks) for weeks 0–28 (E-H). Statistical differences (*; $p < 0.05$) were determined using MANOVA contrasts.

Fig. 8.

Correlations between PCNA/Sox2 and percent response to TCA. Average number of PCNA-expressing cells for each exposure (n = 4–6 tanks; 1 fish per tank) at each time point was plotted against average percent (%) response for TCA behavior tests for each exposure (n = 4–6 tanks; 1 fish per tank) for weeks 0–28 (A–D). Average number of Sox2-expressing cells for each exposure (n = 4–6 tanks; 1 fish per tank) at each time point was plotted against average percent (%) response for TCA behavior tests for each exposure (n = 6–7 tanks) for

weeks 0–28 (E-H). Statistical differences (*; $p < 0.05$) were determined using MANOVA contrasts.

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Fig. 9.

Correlations between calretinin and percent response to TCA. Average integrated density value for calretinin for each exposure ($n = 4-6$ tanks; 1 fish per tank) at each time point was plotted against average percent (%) response for pheromone behavior tests for each exposure ($n = 6-7$ tanks) for weeks 0–28 (A–D). Statistical differences (*; $p < 0.05$) were determined using MANOVA contrasts.