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Fluoxetine treatment ameliorates depression induced by perinatal arsenic exposure via a neurogenic mechanism

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Abstract

Several epidemiological studies have reported an association between arsenic exposure and increased rates of psychiatric disorders, including depression, in exposed populations. We have previously demonstrated that developmental exposure to low amounts of arsenic induces depression in adulthood along with several morphological and molecular aberrations, particularly associated with the hippocampus and the hypothalamic–pituitary–adrenal (HPA) axis. The extent and potential reversibility of this toxin-induced damage has not been characterized to date. In this study, we assessed the effects of fluoxetine, a selective serotonin reuptake inhibitor antidepressant, on adult animals exposed to arsenic during development. Perinatal arsenic exposure (PAE) induced depressive-like symptoms in a mild learned helplessness task and in the forced swim task after acute exposure to a predator odor (2,4,5-trimethylthiazoline, TMT). Chronic fluoxetine treatment prevented these behaviors in both tasks in arsenic-exposed animals and ameliorated arsenic-induced blunted stress responses, as measured by corticosterone (CORT) levels before and after TMT exposure. Morphologically, chronic fluoxetine treatment reversed deficits in adult hippocampal neurogenesis (AHN) after PAE, specifically differentiation and survival of neural progenitor cells. Protein expression of BDNF, CREB, the glucocorticoid receptor (GR), and HDAC2 was significantly increased in the dentate gyrus of arsenic animals after fluoxetine treatment. This study demonstrates that damage induced by perinatal arsenic exposure is reversible with chronic fluoxetine treatment resulting in restored resiliency to depression via a neurogenic mechanism.

Keywords

Arsenic; Psychiatric disorders; Neurogenesis; Fluoxetine; Stress

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

1. Introduction

In a recent report by the ATSDR (Agency for Toxic Substances and Disease Registry), arsenic was singled out as a considerable threat to human health compared to other toxicants, outranking even mercury and lead. While the World Health Organization standard remains at 10 parts per billion (ppb), it is estimated that over 100 million people worldwide are exposed to arsenic in amounts that well exceed this standard (in the ppm range) (Tyler and Allan, 2014). Research over the past few decades has identified pathologies resulting from chronic arsenic exposure, demonstrating that this metalloid disrupts many organ systems in the body, including the brain (Brinkel et al., 2009; Farzan et al., 2013). Epidemiological studies report that cumulative long-term exposure to low doses of arsenic correlates with learning and memory deficits in children and increased rates of depression in adults (Zierold et al., 2004; Rosado et al., 2007; O'Bryant et al., 2011; Roy et al., 2011). Additionally, in almost every country assessed, individuals residing in arsenic-affected areas report a poorer quality of life, poorer mental health, and are more likely to have a mood disorder, such as anxiety or depression, than individuals without significant arsenic exposure (Fujino et al., 2004; Sen and Sarathi Biswas, 2012; Syed et al., 2012).

Research investigating the role of environmental factors, including toxin exposure, in the etiology of depression has gained momentum, as heritability studies report high discordance of the disorder and individual variability in gene expression (Byrne et al., 2013). The early environment during development has been demonstrated to exert profound effects on the modulation of behavior and susceptibility to psychiatric disorders in adulthood (Hochberg et al., 2011). In several rodent studies, pre- and postnatal toxin exposure results in the behavioral endophenotypes of anxiety and depression (Johansson et al., 2007; Onishchenko et al., 2008; Haider et al., 2013). These toxin-induced behaviors are often accompanied by concurrent abnormalities in the regulation of the hypothalamus–pituitary–adrenal (HPA) axis (Hellemans et al., 2008, 2010; Bolton et al., 2013). Individuals with robust homeostatic responses to stress are often considered resistant to depression (Krishnan and Nestler, 2008), thus dysregulation of the HPA axis after toxin exposure may be a mechanism by which toxins, like arsenic, increase susceptibility to psychiatric disorders. Indeed, we have previously demonstrated that perinatal (pre- and postnatal developmental) arsenic exposure (PAE) alters glucocorticoid signaling within the HPA axis, blunts stress responses, increases stress reactivity (Goggin et al., 2012), and results in depressive-like behavior in adulthood (Martinez et al., 2008; Martinez-Finley et al., 2009). In addition to these aberrations, we have also observed altered hippocampal morphology and deficits in adult hippocampal neurogenesis after PAE (Tyler and Allan, 2013). Yet, the pathophysiology of arsenic-induced depression and the response to antidepressants after arsenic exposure has not been fully explored.

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) antidepressant, is the most commonly prescribed treatment for depression. In addition to attenuation of stress-induced depressive-like behaviors, fluoxetine contemporaneously increases adult hippocampal neurogenesis (AHN) in rodents (Wang et al., 2008; Malberg and Duman, 2003; David et al., 2009; Mahar et al., 2014). Impaired AHN has been intimately linked with the onset of depression: recent clinical research reported that depressed patients treated with fluoxetine

do not exhibit hippocampal atrophy or reduced cell numbers as those patients without treatment, further supporting the “neurogenic hypothesis of depression” (Boldrini et al., 2013). Fluoxetine treatment has also been shown to normalize the corticosterone (CORT) response associated with aberrant HPA signaling after prenatal stress (Ishiwata et al., 2005); restoration of HPA axis function is associated with robust increases in neurogenesis in cell culture as well (Anacker et al., 2013). However, the molecular and behavioral responses to fluoxetine in a rodent model with arsenic-induced hippocampal morphological and behavioral deficits have not been determined to date.

To investigate the reversibility of arsenic-induced damage, we evaluated the effect of chronic fluoxetine treatment on behavioral, morphological, and molecular impairments resulting from PAE. To this end, we measured survival and differentiation of neural progenitor cells (NPCs) along with expression of several proteins involved in neurogenesis specifically in the dentate gyrus (DG) to determine fluoxetine’s mechanism of action in arsenic-exposed animals. We further characterized the vulnerability to stress-induced depression generated by arsenic exposure by evaluating behavior and corticosterone (CORT) before and after brief exposure to a predator odor. Chronic fluoxetine treatment reversed deficits in the learned helplessness task; attenuated blunted stress responses and immobility after predator exposure; increased NPC survival and differentiation; and increased expression of brain-derived neurotrophic factor (BDNF), the glucocorticoid receptor (GR), histone deacetylase 2 (HDAC2), and cAMP response element binding protein (CREB) in the DG of arsenic-exposed animals but not in controls. This study suggests that impairments induced by developmental exposure to an environmental toxin like arsenic may be altered with pharmacological intervention in adulthood, leading to normalized hippocampal morphology and behavior.

2. Materials and methods

2.1. Perinatal arsenic exposure paradigm

All procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of New Mexico. Animals were maintained in a 22 °C vivarium on a 12-h reverse light/dark cycle with lights off at 08:00 and *ad libitum* access to water and food. Exposure to arsenic during all three trimesters of brain development was performed as previously described (Tyler and Allan, 2013). Briefly, singly housed female C57BL/6J mice aged 55 days (Jackson Laboratories, Bar Harbor, ME) were acclimated to drinking 50 ppb arsenic-laced water (sodium arsenate, Sigma–Aldrich, St. Louis, MO) for seven to ten days prior to mating. Dams were provided either 50 ppb arsenic water or tap water during breeding and pregnancy until offspring were weaned (Fig. 1A). Offspring were housed two per cage with tap water for the remainder of the study. The number of dams used in this study is reflected in the number of litters provided for each experimental analysis (i.e. $n = 8–10$ indicates that only one animal was used from each of 8 to 10 litters from 8 to 10 different dams). Adult male offspring (postnatal day 70) from dams drinking arsenic water were euthanized by rapid decapitation for tissue dissection or by overdose with sodium pentobarbital followed by transcardiac perfusion with 4% paraformaldehyde for neurogenesis assessment.

2.2. Fluoxetine treatment paradigm and plasma analysis

Fluoxetine hydrochloride (Sigma–Aldrich, St. Louis, MO) was provided for 35 days in drinking water containing 0.066% saccharin to mask any taste of the drug beginning at postnatal day (PD) 35. Male offspring from dams drinking arsenic or tap water were separated into three groups: those drinking 50 mg/L fluoxetine, 100 mg/L fluoxetine, and control (only 0.066% saccharin) as seen in Fig. 1A. Fluoxetine solutions were shielded from the light and changed every other day. Weight gain and fluoxetine ingestion were monitored weekly.

2.3. Predator odor exposure and stress response

Exposure to 2,3,5-trimethyl-3-thiazoline (TMT) (Contech, Victoria, BC, Canada; #300000368) was performed as previously described (Goggin et al., 2012). Animals were individually transferred to an exposure cage containing filter paper saturated with 100 μ L of 3% (v/v) TMT for 10 min. Group-housed animals were exposed concurrently in parallel and returned to home cages for 20 min after the 10-min exposure. Several cohorts of animals were exposed to TMT, some of which were placed into the forced swim task 20 min after exposure, while others were rapidly decapitated for trunk blood collection using Safe-t-Fill EDTA capillary tubes. Samples were centrifuged at $1000 \times g$ for 10 min at 4 °C. Plasma was removed and stored at –80 °C. Corticosterone (CORT) levels in plasma were determined using the DetectX Corticosterone EIA Kit (Arbor Assays, K014-H1) per manufacturer instructions with a 1:100 dilution of plasma prior to analysis. Optical density absorbance was determined by an Infinite M200 instrument and data were analyzed using 4PLC ReaderFit online software.

2.4. Open field

Adult male mice (PD70) were permitted to explore a novel open arena, measuring 40 cm $\times$ 40 cm $\times$ 35 cm and constructed of white Plexiglas, for 15 min for two consecutive days. Dim red overhead lighting was used along with white spotlights to illuminate the corners of the open field at 45–60 lumen and the center at 75–90 lumen. Total distance traveled was measured with the aid of an Ethovision video tracking system using the coordinates of the animal's center of mass image compared against a template of the arena dimensions. Locomotor activity in a novel environment was assessed the first day, and locomotor activity in a familiar environment was assessed on the second day.

2.5. Forced swim

The forced swim task (FST) was conducted essentially as previously described (Caldwell et al., 2008; Martinez et al., 2008) with a critical difference in the time of behavior assessment. Our prior studies used a conventional light/dark cycle (lights *on* at 08:00); however, our current studies use a reverse light/dark cycle (lights *off* at 08:00). Thus, all behavioral assessments occurred during the nocturnal (active) phase of the animals' circadian cycle (Kelliher et al., 2000). Adult male mice (PD70) were placed in a clear Plexiglas cylinder (46 cm height $\times$ 30 cm diameter) filled with warm water (27 °C) to a depth such that the animal's tail did not touch the bottom of the container. Both latency to cease active swimming and total immobility (cessation of escape directed movement) were scored for 5

min by an experimenter blind to group designations (Porsolt et al., 1977). The forced swim test was conducted with and without exposure to predator odor in separate cohorts of animals 20 min after cessation of the TMT exposure.

2.6. Learned helplessness

The learned helplessness (LH) task was conducted essentially as previously described (Caldwell et al., 2008; Martinez et al., 2008) with critical differences including the time of behavioral assessment and a more mild inescapable shock training. As previously stated, all behavioral assessments were performed during the nocturnal (active) phase of the animals' circadian cycle, as this has been shown to affect stress-related behavior. Animals were trained in the Coulbourn™ Habitest© shuttle box apparatus with 20 unpredictable foot shocks (0.5 mA, 2-s) over the course of 10 min for one session. Twenty-four hours later, animals were placed in the shuttle box and given 28 trials of shocks with a 30-s intertrial interval. Escape latency was measured as the time between opening and closing of the shuttle box door. The criterion for determining the number of trials to acquisition was set as three consecutive escape latencies of 10 s or less. Failure to escape after 24 s resulted in shock termination and door closure.

2.7. Assessment of adult neurogenesis and confocal stereology

Differentiation and survival of neural progenitor cells (NPC) in the dentate gyrus was assessed as previously described (Tyler and Allan, 2013). Briefly, 5-bromo-2'-deoxyuridine (97% Sigma–Aldrich, St. Louis, MO) BrdU was used to label NPCs in a pulse-chase assay using four injections (I.P.) of 50 mg/kg BrdU over a 12-h period at PD40. Thirty days later, coronal sections (40 µm) of the rostral–caudal extent of the hippocampus were obtained from 4% paraformaldehyde perfused adult male mice (PD70). Immunohistochemistry was performed for 1:6 serial sections over the dorsal extent of the dentate gyrus. Free-floating sections were incubated for 72 h in primary antibodies for BrdU (1:300; Abcam, ab6326) and NeuN (1:1500; Millipore, MAB377) and subsequently incubated in secondary antibodies Alexa 488 (1:500; Invitrogen, A11006) and Alexa 647 (1:500; Invitrogen, A21236) and DAPI (1:300 nM; Sigma) for 2 h at room temperature. Colocalization of BrdU and NeuN was determined for measurement of differentiated NPCs, while BrdU-only labeled cells were indicative of survival of NPCs. While it should be noted that some BrdU cells might be of glial origin (Steiner et al., 2004), this approach is congruent with other recent studies assessing differentiation and survival (Klempin et al., 2010; Hamilton et al., 2014; Jiang et al., 2014). Confocal stereology was performed using an Olympus DSU spinning disk inverted IX-81 microscope; a 40× objective through the orthogonal plane of the tissue was used to visualize colocalization of markers. Quantification of the number of cells with colocalization for 10 slices per brain was performed using StereoInvestigator software; specifications include a guard zone of 2 µm and a probe of 20 µm. Images for publication were acquired using a Zeiss LSM510 META confocal microscope with a 100× oil objective or a 10× objective to obtain maximum intensity projections of Z-stacks.

2.8. Protein expression quantification via immunoblotting

Immunoblotting was conducted essentially as previously described using established protocols (Caldwell et al., 2014). Microdissected dentate gyrus tissue derived from one

animal (Hagihara et al., 2009) was homogenized in a Biomasher II disposable microhomogenizer (Kimble Chase) with homogenization buffer containing 20 mM Tris-HCl, 1 mM EDTA, 320 mM sucrose, protease inhibitor cocktail (1:1000; Sigma, P8340), and 200 μ M sodium orthovanadate, pH 7.4. The nuclear fraction was obtained via centrifugation at $1000 \times g$ for 6 min at 4 °C followed by resuspension in homogenization buffer and further centrifugation at $1000 \times g$ for 10 min; the supernatant was collected as the post-nuclear lysate and stored at -80 °C. The nuclear pellet was resuspended in homogenization buffer containing 1% (v/v) Triton X-100, 75 mM NaCl, and 75 mM KCl; the nuclear envelope was lysed via sonication and centrifuged at $15,000 \times g$ for 10 min. The supernatant was collected as the nuclear fraction and stored at -80 °C. Target proteins were assessed in the nuclear (CREB: 20 μ g, GR: 10 μ g, HDAC2: 10 μ g) and post-nuclear (BDNF: 21 μ g) subcellular fractions of the dentate gyrus. Samples from each of four groups (arsenic and control animals drinking only 0.066% saccharin solution or 100 mg/L fluoxetine and 0.066% saccharin solution) were run on the same blots for each target protein. Samples ($n = 7-10$ per group) were loaded in NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, NP0336), separated by electrophoresis, and transferred to PVDF membranes (Millipore Corporation, IPFL00010). Membranes were incubated overnight using the following primary antibodies in 1:1000 dilutions: anti-CREB (Cell Signaling, 9104), anti-GR (Santa Cruz, sc-1004), anti-HDAC2 (Santa Cruz, sc-81599), and anti-BDNF (Santa Cruz, sc-546). All primary antibodies were diluted in PBS-T (1.06 mM KH_2PO_4 , 2.97 mM Na_2HPO_4 , 155 mM NaCl, 0.1% (v/v) Tween-20, pH 7.4). Membranes were incubated for 45 min in respective secondary antibodies dilute to 1:15,000 in PBS-T: IRDye 680RD (LiCOR Biosciences, 926-68073) and IRDye 800CW (LiCOR Biosciences, 926-32212). Quantification of immunoreactivity was performed using two-channel infrared detection Odyssey[®] Imaging System (LI-COR Biosciences); Image Studio (LI-COR Biosciences, version 3.1) was used for quantification assessment. For normalization, all blots were stained with IRDye[®] Blue Protein Stain (LI-COR Biosciences, 3343C056); Coomassie staining was demonstrated to be linear within the range of protein concentrations used in these studies. All blots were normalized to their respective Coomassie stain for quantification.

2.9. Statistical analysis

Global analysis of variance (ANOVA) for two factors (arsenic, fluoxetine) was used to assess all data; further analysis of significant main effects was performed using post hoc Student's *t*-test with Bonferroni correction. Homogeneity of variance was confirmed using Levene's test. Data are presented as mean $\pm$ SEM, and *p* value <.05 was deemed statistically significant for all experiments. The reported *n* refers to the number of litters used in each experiment, with only one animal per litter used in each experimental group to avoid litter effects. Separate animals were used for each behavioral experiment, and tissue derived from animals without behavioral assessment was used for all molecular and neurogenesis studies. All studies contain at least three different breeding rounds per assay; $n = 7-11$ and is reported for each assay.

3. Results

3.1. Perinatal arsenic exposure does not interfere with consumption of fluoxetine in adulthood

Fluoxetine consumption was monitored every other day for five weeks (Fig. 1A) beginning on PD35. Average intake was calculated for each group: 9.79 ± 0.21 and 9.97 ± 0.21 mg/kg/day for arsenic and control groups consuming 50 mg/L fluoxetine, and 16.6 ± 0.66 and 17.1 ± 0.47 mg/kg/day for arsenic and control groups consuming 100 mg/L fluoxetine. No differences among exposure groups in consumption of fluoxetine were found (Fig. 1B). Weight gain was monitored weekly in all animals; no differences in weight were observed among exposure or treatment groups (Fig. 1C). Average weight gain per week was 1.44 ± 0.13 mg/week/animal ($n = 8$ –10 animals per group where n = number of litters).

3.2. Fluoxetine treatment prevents depressive-like symptoms induced by perinatal arsenic exposure in a mild learned helplessness task

We have previously demonstrated that arsenic induces depressive-like symptoms in a robustly stressful learned helplessness (LH) task consisting of one hour of training with 120 uncontrollable foot shocks. In this experiment, we sought to assess the response to mild stress, which does not result in depressive-like symptoms in control animals. To validate the construct validity of such a task, chronic fluoxetine treatment (for one month prior to the LH task) was used to assess if depressive-like symptoms could be prevented. A mild learned helplessness task consisting of 20 uncontrollable foot shocks given over 10 min and subsequent assessment of latency 24 h later, revealed a significant main effect of arsenic ($F_{(1,47)} = 6.58$; $p = .01$) and of fluoxetine ($F_{(2,47)} = 9.19$; $p = .0004$) with a significant interaction ($F_{(2,47)} = 7.24$; $p = .002$). Results are provided graphically in Fig. 2A for average escape latency and in Fig. 2B for trials to acquisition. Average escape latency for arsenic-exposed animals was 10.4 ± 1.8 s which was reduced by 52% with fluoxetine treatment (4.9 ± 0.6 s), ($p < .001$) independent of dose (50 mg/L versus 100 mg/L). Average escape latency for control animals was 5.4 ± 0.4 s (a 48% reduction compared to arsenic-exposed animals, $p < .01$) with no effect of fluoxetine consumption, suggesting lack of depressive-like symptoms. Assessment of trials to acquisition suggests a similar pattern: significant main effects of arsenic ($F_{(1,47)} = 4.39$; $p = .03$) and of fluoxetine ($F_{(2,47)} = 8.77$; $p = .0006$) with a significant interaction ($F_{(2,47)} = 4.28$; $p = .02$) yet no differences among fluoxetine treatment groups in control animals. Due to a lack of dose-dependent effects, all subsequent treatments used 100 mg/L fluoxetine in drinking water. ($n = 8$ –10 animals per group where n = number of litters).

3.3. Fluoxetine treatment prevents depressive-like behavior induced by perinatal arsenic exposure after TMT predator odor exposure in the forced swim task

We sought to assess stress-induced depressive-like behavior during the active nocturnal cycle in a forced swim task (FST) using warm water (25–27 °C) to prevent the hypothermia confound observed in several FST paradigms (Petit-Demouliere et al., 2005). We have previously shown adult males perinatally exposed to arsenic exhibit depressive-like behaviors during the diurnal cycle. To determine the resiliency of arsenic-exposed animals and the efficacy of fluoxetine, we assessed depressive-like symptoms in the forced swim

task 20 min after a 10-min exposure to a predatory odor (trimethylthiazoline, TMT, the main component of fox urine) for which we have previously shown arsenic-exposed animals do not respond appropriately. Results are provided graphically in Fig. 2C for total immobility and in Fig. 2D for latency. Analysis of immobility revealed no significant main effects; however, post hoc analysis indicates that arsenic exposure increases immobility ($p < .01$) and treatment with 100 mg/L fluoxetine ameliorates this depressive-like behavior in the arsenic-exposed group ($p < .001$). Measurement of latency to cease active swimming revealed a significant main effect of arsenic ($F_{(1,28)} = 12.7$; $p = .001$), no significant main effect of fluoxetine, and an interaction ($F_{(1,28)} = 10.99$; $p = .0025$), suggesting that arsenic reduces resiliency in the FST. Fluoxetine treatment (100 mg/L) significantly increased latency in arsenic-exposed animals ($p < .05$) indicating restoration of resiliency, but had no effect on control animals ($n = 8$ –10 animals per group where n = number of litters).

3.4. Neither perinatal arsenic exposure nor fluoxetine treatment alters locomotion in the open field task

To determine if altered behavior in the LH and FST behavioral tasks was due to impairment of locomotion, we assessed total movement in the open field test (OFT). Data analysis indicated no significant main effects of arsenic or fluoxetine at PD70 on Day 1 of the OFT. Total movement decreased in all groups on Day 2 of the OFT as expected (data not shown) ($n = 8$ –10 animals per group where n = number of litters).

3.5. Fluoxetine treatment restores the blunted stress response to predator odor induced by perinatal arsenic exposure

We have previously demonstrated that arsenic-exposed mice fail to activate a normal corticosterone (CORT) response to TMT (Goggin et al., 2012); to determine if fluoxetine attenuates this effect, CORT levels at baseline and after TMT exposure were determined in all animals. As seen in Fig. 2E, perinatal arsenic exposure increases the baseline levels of CORT: 45.3 ± 3.2 ng/ml compared to 28.8 ± 5.6 ng/ml in control animals ($p < .05$) as expected. Fluoxetine treatment did not alter baseline CORT levels in arsenic-exposed animals or in control animals. Plasma was obtained 30 min after the onset of a 10-min TMT exposure; subsequent analysis of change in CORT levels was determined using average baseline amounts ($\text{CORT}_{\text{TMT}}/\text{CORT}_{\text{Baseline}}$) as seen in Fig. 2F. Two-way ANOVA analysis indicated no significant main effects of arsenic or fluoxetine, but a significant interaction ($F_{(1,27)} = 10.97$; $p = .003$). Perinatal arsenic exposure decreased the CORT response compared to controls ($p < .01$). Treatment with fluoxetine did not impact control animals and increased the CORT response in arsenic-exposed animals up to levels measured in the control animals ($p < .01$). These data suggest that arsenic-exposed animals have a blunted but prolonged response to TMT (stress) and fluoxetine treatment attenuates this effect ($n = 7$ –10 animals per group where n = number of litters).

3.6. Fluoxetine treatment ameliorates deficits in survival and differentiation of neural progenitor cells after perinatal arsenic exposure

To determine if chronic treatment with 100 mg/L fluoxetine induces alterations in adult neurogenesis in adult males perinatally exposed to arsenic, the number of BrdU+ and BrdU

+NeuN+ cells were measured four weeks after a pulse chase BrdU assay. Fig. 3A shows triple staining (DAPI+BrdU+NeuN+) used for assessment of the number of differentiated neural progenitor cells (NPCs). All cells containing BrdU were counted for assessment of survival of NPCs. Fig. 3D and 3F are representative images from arsenic-exposed animals at PD70 with and without five weeks of fluoxetine treatment, respectively. Fig. 3E and 3G are representative images from control animals at PD70 with and without five weeks of fluoxetine treatment, respectively. For the number of BrdU+NeuN+ cells, two-way ANOVA data analysis indicates significant main effects of arsenic ($F_{(1,26)} = 9.675$; $p = .005$) and fluoxetine ($F_{(1,26)} = 9.82$; $p = .004$) but no significant interaction. Arsenic-exposed animals had 654 ± 60 BrdU+NeuN+ labeled cells; fluoxetine exposure resulted in 1016 ± 37 BrdU+NeuN+ labeled cells, a significant increase of 55% ($p < .05$). Control animals had 1014 ± 52 BrdU+NeuN+ labeled cells (also a 55% increase over arsenic-exposed animals $p < .01$); fluoxetine resulted in 1167 ± 144 BrdU+NeuN+ labeled cells in control animals which was not a significant increase. A similar trend was observed for BrdU+ cells: two-way ANOVA analysis revealed a significant main effect of arsenic ($F_{(1,28)} = 8.33$; $p = .007$) and fluoxetine ($F_{(1,28)} = 13.9$; $p = .001$) but no significant interaction. Arsenic-exposed animals had 843 ± 68 BrdU+ labeled cells, significantly 53% less than the 1240 ± 78 BrdU+ labeled cells in control animals ($p < .05$). Fluoxetine treatment in arsenic-exposed animals resulted in a 57% increase ($p < .001$), 1325 ± 36 BrdU+ labeled cells. Fluoxetine treatment increased the number of BrdU+ cells by 22% (1511 ± 169 BrdU+ labeled cells) in control animals but this effect was not significant ($n = 7-9$ animals per group where n = number of litters).

3.7. Fluoxetine treatment increases expression of several proteins associated with neurogenesis in the dentate gyrus after perinatal arsenic exposure

To determine the mechanism by which fluoxetine rescues deficits in survival and differentiation of NPCs in perinatally arsenic exposed animals, protein expression of several factors that contribute to adult neurogenesis was assessed in dentate gyrus tissue. As we have previously demonstrated reduced expression of the glucocorticoid receptor (GR) in the hippocampus of PD35 arsenic-exposed animals (Martinez-Finley et al., 2009), GR expression in the nuclear fraction of the dentate gyrus at PD70 was assessed. Representative blots and data are shown in Fig. 4A for the GR. There were no significant main effects of arsenic but a significant main effect of fluoxetine ($F_{(1,38)} = 8.5$; $p = .006$) and no significant interaction; however, treatment with fluoxetine increased expression of the GR in both arsenic-exposed and control groups ($p < .05$, Fig. 4A). Histone deacetylase 2 (HDAC2) has been implicated in depression and has a known GR response element in its promoter. Perinatal arsenic exposure did not alter protein quantification of HDAC2 in the nuclear fraction of the dentate gyrus (Fig. 4B); however, there was a significant main effect of fluoxetine treatment ($F_{(1,36)} = 6.1$; $p = .02$) with significantly increased expression of HDAC2 in arsenic-exposed animals ($p < .05$) but not in control animals. Brain derived neurotrophic factor (BDNF) is regulated by the GR, HDAC2, and cAMP response element binding protein (CREB); both BDNF and CREB are paramount to proper maturation and differentiation of neural progenitor cells (Gass and Riva, 2007). Protein quantification of the mature form of BDNF in the post-nuclear lysate fraction of the DG and of CREB in the nuclear fraction of the DG was determined. Representative blots and data are shown in Fig. 4C for BDNF and Fig. 4D for CREB. A significant main effect of fluoxetine on BDNF was

determined ($F_{(1,36)} = 6.2$; $p = .012$). While PAE did not alter BDNF expression, arsenic-exposed animals treated with fluoxetine had higher immunoreactivity to BDNF than their non-treated counterparts ($p < .05$), with no effect on control animals. A similar pattern was observed for CREB protein expression: a significant main effect of fluoxetine was determined ($F_{(1,31)} = 8.5$; $p = .0065$), with a significant difference among arsenic-exposed animals with and without fluoxetine treatment ($p < .05$) but not among control animals ($n = 8$ – 11 animals per group where n = number of litters).

4. Discussion

Toxins such as lead, methylmercury, organophosphates, and arsenic have been associated with psychiatric disorders, including depression, in a number of epidemiological studies (Zierold et al., 2004; Sen and Sarathi Biswas, 2012; Beard et al., 2013; Freire and Koifman, 2013; Malekiran et al., 2013; Ojo et al., 2014; Weisskopf et al., 2013). Yet, the impact of environmental factors, particularly toxins, on the susceptibility to depression has not been investigated in great detail. We have previously shown several behavioral deficits, morphological abnormalities, and molecular aberrations induced by developmental arsenic exposure in adult mice (Martinez et al., 2008; Martinez-Finley et al., 2009, 2011; Tyler and Allan, 2013). In the present study, we determined the extent to which chronic fluoxetine treatment was able to reverse these effects and restore resiliency to stress-induced depression in arsenic-exposed animals.

Animals perinatally exposed to arsenic likely exhibit depressive-like symptoms and negatively process congruent information due to hyperactivity of the HPA axis, as observed in depressed patients and other rodent models (Vollmayr and Gass, 2013). Here, we have demonstrated that exposure to mild stress on the first day of a learned helplessness (LH) task was sufficient to induce depressive-like symptoms in arsenic-exposed mice, but not in controls, suggesting a lack of resiliency to stress. This particular LH paradigm is minimal (low foot shock and few inescapable exposures) such that it is unlikely to induce depressive-like behaviors in controls as other LH paradigms do. Additionally, arsenic-exposed mice exhibit depressive-like behavior in the forced swim task (FST) after a 10-min exposure to predator odor, while control animals do not. This effect may be mediated through alterations in the HPA axis signaling leading to an aberrant stress response. To support this assertion, we determined that corticosterone (CORT) levels are elevated under basal conditions in arsenic-exposed animals; and while hyperlocomotion due to elevated CORT can confound activity measured in depression tasks (Strekalova et al., 2005), we found no difference in total locomotor activity in the open field test (data not shown). The blunted CORT response we measured after predator odor exposure in arsenic-exposed animals is conceivably due to the elevated CORT before the stressor and the lack of sufficient CORT activation. Reduced HPA axis responses have been observed in animals prenatally exposed to stress with behavioral deficits in adulthood due to HPA hyperactivity (Darnaudery and Maccari, 2008). Furthermore, abnormal HPA axis function has been measured in depressed patients, along with increased release of cortisol and blunted HPA axis feedback; these alterations are diminished after chronic treatment with antidepressants, typically preceding alleviation of behavioral symptoms (Wainwright and Galea, 2013). Thus, perturbation the HPA axis may be required to elucidate distinctions in depressive-like behavior between arsenic and control

animals, yet these behaviors may be impermanent as resiliency to depressive-like symptoms was restored after chronic treatment with fluoxetine in a dose-independent manner.

Recent studies have demonstrated a link between the early environment, development of HPA axis feedback, and adult hippocampal neurogenesis (Loi et al., 2014). Results presented here suggest that perinatal arsenic exposure induces unambiguous but reversible deficits in adult neurogenesis by reducing the survival (BrdU+ cells; 53% less) and differentiation (BrdU+NeuN+; 55% less) of neural progenitor cells four weeks after BrdU injection at PD70. Chronic treatment with 100 mg/L fluoxetine, resulting in 17 mg/kg/day consumption, attenuated these deficits in the arsenic-exposed animals: we measured a 57% increase in survival of BrdU+ cells and a 55% increase in the number of differentiated BrdU+NeuN+ cells. Region specificity was not determined; however all measurements occurred in the dorsal hippocampus. Additionally, BrdU+ cells may include a small population of glial cells, but this effect may be negligible (Steiner et al., 2004). These results concur with our previous observations of the robust effect of intermittent interval exposure to enrichment on neurogenesis after arsenic exposure (Tyler and Allan, 2013). This increase in adult neurogenesis coincident with restored resiliency to stress-induced depression suggests that fluoxetine is ameliorating arsenic-induced deficits via a neurogenic mechanism.

Recent genomic analysis has demonstrated that genes associated with adult hippocampal neurogenesis are impacted by SSRI treatment (Malki et al., 2012). Additionally, HPA axis stress responses in the dentate gyrus are mediated in part through the glucocorticoid receptor (GR) and can impact gene expression (de Kloet et al., 2005). For its part, arsenic exposure induces deficits in mRNA expression of neurogenesis-related genes, including *Nrc3l* (gene for GR protein), *Creb1*, and *Hdac2* (Goggin et al., 2012; Tyler and Allan, 2013), though the mechanism by which this damage occurs is unclear. Thus to determine the pathways involved in fluoxetine's neurogenic actions and in arsenic-induced morphological damage, we assessed expression of proteins involved in AHN, including the GR, CREB, HDAC2, and BDNF. We would have expected all of these proteins to be reduced in arsenic-exposed animals, but arsenic exposure did not result in altered levels for any of these proteins. Our results may be specific to the dentate gyrus: studies demonstrating reduced CREB and BDNF, in response to either prenatal stress or toxins such as lead or mercury, have done so using mRNA expression in the dentate gyrus (similar to our previous observations) or protein quantification in the whole hippocampus, but never protein expression in the dentate gyrus (Onishchenko et al., 2008; Feng et al., 2012; Boersma et al., 2014; Guan et al., 2013; Sanchez-Martin et al., 2013). While arsenic exposure in the parts-per-million range has been shown to reduce CREB and associated proteins (Wang et al., 2009), the arsenic concentration in our study is in the parts-per-billion range, closer to the levels humans are exposed to, and likely too low to impair CREB signaling. Additionally, reduced *Nr3c1* and *Hdac2* mRNA levels in the hippocampus or reduced *Creb1* mRNA in the dentate gyrus may not translate to reduced protein levels, as seen in several other studies. While these proteins may not be altered, their downstream signaling, including ERK from the BDNF pathway, may lead to deficits in AHN or lead to greater susceptibility to stress-induced depression (Numakawa et al., 2013). Thus, reduced AHN in arsenic-exposed animals does not derive

from alteration of these target proteins, and elucidating an arsenic-specific mechanism requires further investigation.

While arsenic does not impact the expression of CREB, HDAC2, GR, or BDNF, chronic fluoxetine treatment increases expression of all target proteins, particularly in the arsenic-exposed animals. These results suggest that the mechanism by which fluoxetine enhances AHN is separate from the pathways damaged by arsenic exposure. Increased signaling of 5-HT receptors after fluoxetine administration ultimately leads to a steady increase in cAMP, protein kinase A, and CREB (Pinnock et al., 2010). CREB, a histone acetyltransferase, then allows for increased transcription of BDNF, which has antidepressant effects when released and feedback on CREB. Thus, increased BDNF and CREB expression in the dentate gyrus after fluoxetine treatment was expected in these studies, as others have demonstrated similar results, particularly in rodent models with impaired stress responses (Blom et al., 2002; Vinet et al., 2004). Fluoxetine also enhances functionality of the GR (Pariante et al., 2003) allowing for termination of HPA signaling via improved negative feedback (Sapolsky et al., 1984), as seen in studies presented here using the predator odor and FST challenge. This enhanced GR signaling directly increases AHN (Pariante et al., 2003; Anacker et al., 2011) and uses neurogenesis-dependent mechanisms to restore control over the HPA after dexamethasone administration (Surget et al., 2011). The GR has also been demonstrated to increase expression of HDAC2 via the *Hdac2*-GRE (Graff et al., 2012), while others have shown HDAC2 is necessary for GRE-mediated gene activation (Luo et al., 2009). However, HDAC2 negatively regulates BDNF (Guan et al., 2009), though we are the first to show these alterations specifically in the dentate gyrus; as such, the interaction among these proteins after fluoxetine treatment remains to be elucidated. Overall, fluoxetine treatment is ameliorating depressive-like behavior after PAE by enhancing AHN in the dentate gyrus likely mediated by these increased proteins. This effect is particularly robust in arsenic-exposed animals, as neither protein expression nor AHN was significantly increased in control animals after fluoxetine treatment.

A recent study reported that fluoxetine does not increase BDNF levels in males after chronic psychosocial isolation stress (Mitic et al., 2013), and several others have demonstrated that in control animals not exposed to stress, BDNF does not increase after fluoxetine treatment (Nibuya et al., 1995; Dias et al., 2003; De Foubert et al., 2004). While we anticipated increased protein expression and AHN in control animals after fluoxetine treatment, our results are consistent with these studies and the lack of altered behavior and stress response we observed in control animals. However, AHN is a sensitive system easily influenced by experience, stress, and pharmacological manipulation. As such, it is likely that our findings for control animals can be attributed to the method of fluoxetine administration. A similar pattern was reported in animals consuming fluoxetine via drinking water after developmental methylmercury exposure: three weeks of chronic fluoxetine treatment (80 mg/L) ameliorated deficits in total neuronal number in exposed animals but did not significantly impact cell numbers in unexposed control animals (Bose et al., 2012). Other delivery methods of fluoxetine generate different results: animals given 18 mg/kg fluoxetine via gavage for 28 days exhibit increased proliferation and maturation of cells in the hippocampus (Wang et al., 2008), while interperitoneal (IP) administration of 5 or 10

mg/kg/day fluoxetine for 28 days increases survival of neural progenitor cells one month (Malberg et al., 2000) and 24 h after BrdU injection (Santarelli et al., 2003) in control animals, respectively. Both the IP and the gavage procedures provide instant delivery of the antidepressant, which should accelerate the time to achieving peak blood levels and may affect downstream mechanisms. It should also be noted that both methods of administration (injection and gavage) are stressful and may reduce AHN in the vehicle control animals not provided antidepressants. Similarly, fluoxetine increases proliferation of NPCs after mild chronic stress (Surget et al., 2011) and survival and maturation of NPCs after corticosterone injection (David et al., 2009) but this effect is not observed in control animals without exposure to stress. Since arsenic-exposed animals have alterations in the hypothalamic–pituitary–adrenal-axis (HPA) resulting in aberrant stress signaling (Goggin et al., 2012), we sought to provide the antidepressant using the least stressful method.

The evidence provided here strengthens the connection between AHN and depression, as arsenic-exposed mice have decreased AHN and depressive-like symptoms; however, this association is correlational. It is unknown if alterations in the HPA axis result in deficits in AHN that ultimately lead to the onset of depression or if altered AHN is a byproduct of stress-induced depression (David et al., 2009; Anacker et al., 2013). In this study, increased AHN via fluoxetine in arsenic-exposed animals is concurrent with increased resiliency to stress-induced depression in the mild stressor LH task and in the FST following predator odor exposure. This effect may be mediated by increased AHN, possibly via increased nuclear GR in the dentate gyrus, as demonstrated in the hippocampus in other studies (Anacker et al., 2011), or via reduced basal levels of CORT and a subsequent proper response to stress. The serotonergic system may also contribute to the efficacy of fluoxetine: arsenic-exposed mice have decreased serotonin levels and increased 5HT_{1A} receptors in the dorsal hippocampus (Martinez et al., 2008), and these receptors have been implicated in the efficacy of fluoxetine as an antidepressant (Riad et al., 2004). It is likely that fluoxetine alters morphology, behavior, and molecules in the arsenic-exposed animals via all of these pathways: increased AHN, restored stress response, and serotonergic signaling via 5HT_{1A} receptors, although we have yet to measure the latter.

5. Conclusion

Several epidemiological and rodent studies have demonstrated a correlation between exposures during gestation or early development and increased risk for developing psychiatric disorders (Hellemans et al., 2010; Davis et al., 2013; Talati et al., 2013); indeed, arsenic exposure has been associated with depression in epidemiological studies (Zierold et al., 2004). While it is unclear if the damage resulting from developmental exposures is amenable to intervention, we have provided evidence that arsenic-induced deficits can be reversed with fluoxetine treatment. Using a perinatal arsenic exposure model in mice, we have demonstrated that arsenic reduces resiliency to stress-induced depression, induces a blunted CORT response, and decreases adult hippocampal neurogenesis. These effects were ameliorated and behavior restored with chronic fluoxetine treatment, along with increased levels of proteins associated with neurogenesis in the dentate gyrus. Based on these findings, fluoxetine restores resiliency to depression via a neurogenic mechanism. However, the mechanisms through which arsenic exposure reduces AHN are likely not the same

mechanism by which fluoxetine reverses these effects. While fluoxetine increases expression of neurogenesis-related proteins, deficits in these proteins were not found after arsenic exposure. Thus, fluoxetine is likely bypassing arsenic-induced damage to increase AHN. As such, while alterations in morphology and behavior after PAE are ameliorated by chronic fluoxetine treatment, the mechanism by which arsenic produces deficits in AHN is still under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neuro.2014.06.006>.

Fig. 1.

Perinatal arsenic exposure paradigm followed by chronic fluoxetine treatment. (a) Dams were provided water with 50 parts-per-billion (ppb) arsenic 10 days prior to and throughout breeding and pregnancy until weaning of offspring at postnatal day 23 (PD23). Offspring were placed on tap water until PD35. Male offspring were split into 6 groups: arsenic-exposed or control animals consuming either 50 mg/L fluoxetine with 0.066% saccharin, 100 mg/L fluoxetine with 0.066% saccharin, or 0.066% saccharin only for 35 days (approximately PD35-PD70). BrdU (5-bromo-2'-deoxyuridine) was used to label neural progenitor cells in a pulse-chase assay using four injections (I.P.) of 50 mg/kg BrdU over a 12-h period at PD40. Assessments of neurogenesis, behavior, and protein expression were

conducted at PD70. (b) Male offspring from either the arsenic-exposed or control groups consume the same quantities of fluoxetine in drinking water. Average consumption for the arsenic-exposed offspring was 9.79 ± 0.21 mg/kg/day and 16.6 ± 0.66 mg/kg/day for the 50 mg/L and 100 mg/L fluoxetine groups, respectively. Average consumption for the control groups was not significantly different from arsenic-exposed groups: 9.97 ± 0.21 mg/kg/day and 17.1 ± 0.47 mg/kg/day for the 50 mg/L and 100 mg/L fluoxetine groups, respectively. (c) Fluoxetine consumption did not alter weight gain in the exposure groups; weight gain was monitored weekly. All animals were approximately the same weight (after week 4) during assessments.

Learned Helplessness

Forced Swim

Stress Response

Fig. 2.

Chronic fluoxetine treatment enhances the resiliency to stress-induced depression and restores the stress response in arsenic-exposed animals at PD70. (a) Perinatal exposure to arsenic increases escape latency in the learned helplessness (LH) task at PD70 ($p < .01$). Chronic treatment with 50 mg/L or 100 mg/L fluoxetine decreases latency in arsenic-exposed animals ($p < .001$) in a dose-independent manner. Escape latency of control animals with and without fluoxetine treatment was not altered in this mild LH task. (b) Perinatal arsenic exposure increases the number of trials to criteria in the LH task at PD70 ($p < .01$). The criterion for determining the number of trials to acquisition was set as three consecutive

escape latencies of 10 s or less. Chronic treatment with 50 mg/L or 100 mg/L fluoxetine decreases the number of trials to acquisition in arsenic-exposed animals ($p < .01$) in a dose-independent manner. Average number of trials to acquisition for control animals with and without fluoxetine treatment was not altered in this mild LH task. (c) Total immobility in the forced swim test (FST) of arsenic-exposed animals is increased compared to controls ($p < .01$) 30 min after the onset of a 10-min predator odor exposure (TMT). Chronic treatment with 100 mg/L fluoxetine decreased immobility time of arsenic-exposed animals ($p < .001$), but had no effect on immobility time of control animals. (d) Latency to immobility in the FST is decreased in arsenic-exposed animals compared to controls 30 min after the onset of a 10-min predator odor exposure (TMT) with a significant main effect of arsenic ($F_{(1,28)} = 12.7$; $p = .001$). Chronic treatment with 100 mg/L fluoxetine increased latency to immobility of arsenic-exposed animals ($p < .05$), but had no effect on the latency duration in control animals. (e) Basal corticosterone (CORT) levels in serum are elevated in arsenic-exposed animals at PD70 compared to controls ($p < .05$). Chronic treatment with 100 mg/L fluoxetine had no effect on basal CORT levels in either perinatal exposure group. (f) The percent CORT response, measured as CORT levels 30 min post-onset of the 10-min exposure to TMT divided by the basal CORT levels, was reduced by perinatal arsenic exposure ($p < .01$). Chronic treatment with fluoxetine restored the percent CORT response ($p < .01$) in arsenic-exposed animals, but had no effect on control animals. *** $p < .001$, ** $p < .01$, * $p < .05$.

Fig. 3.

Chronic fluoxetine treatment restores deficits in adult hippocampal neurogenesis in arsenic-exposed animals at PD70. (a) Representative images of the dentate gyrus were taken with 100× objective to demonstrate colocalization of markers to identify neurons (BrdU+NeuN+) and neural progenitor cells (BrdU+). NeuN (red) mature marker of neurons; DAPI (blue) nuclear counterstain; BrdU (green) exogenous mitotic marker; Merge, all three markers (b). Perinatal arsenic exposure decreases the number of BrdU+NeuN+ cells in the dentate gyrus compared to controls at PD70 ($p < .01$). Chronic treatment with 100 mg/L fluoxetine increases the number of BrdU+NeuN+ cells in arsenic-exposed animals ($p < .05$) but not in controls. (c) Perinatal arsenic exposure decreases the number of BrdU+ cells in the dentate gyrus compared to controls at PD70 ($p < .05$). Chronic treatment with 100 mg/L fluoxetine increases the number of BrdU+ cells in arsenic-exposed animals ($p < .05$) but not in controls. *** $p < .001$, ** $p < .01$, * $p < .05$. The following representative images were taken with 10× objective on a META confocal microscope. NeuN (red) mature marker of neurons; DAPI (blue) nuclear counterstain; BrdU (green) exogenous mitotic marker; Merge, all three markers represented. (d) Representative image of the dentate gyrus at PD70 from an arsenic-exposed animal. (e) Representative image of the dentate gyrus at PD70 from a control animal. (f) Representative image of the dentate gyrus at PD70 from an arsenic-exposed

animal chronically treated with 100 mg/L fluoxetine. (g) Representative image of the dentate gyrus at PD70 from a control animal chronically treated with 100 mg/L fluoxetine.

Fig. 4.

Chronic fluoxetine treatment increases expression of proteins associated with neurogenesis in the dentate gyrus of arsenic-exposed animals at PD70. (a) In (A)–(D), representative images of immunoblots are shown for arsenic-exposed and control animals consuming 0.066% saccharin or 100 mg/L fluoxetine with 0.066% saccharin. The immunoreactivity for each protein antibody was normalized to its respective Coomassie stain for quantification (see Supplementary Figure). (b) Perinatal arsenic exposure does not alter the protein expression of the glucocorticoid receptor (GR) in the nuclear fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of the GR in both arsenic exposed animals ($p < .05$) and control animals ($p < .05$) at PD70. (c) Perinatal arsenic exposure does not alter the protein expression of histone deacetylase 2 (HDAC2) in the nuclear fraction of the dentate gyrus at PD70 compared to

controls. Chronic treatment with 100 mg/L fluoxetine increases expression of HDAC2 in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. (d) Perinatal arsenic exposure does not alter the protein expression of the mature form of brain-derived neurotrophic factor (BDNF) in the post-nuclear lysate fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of mature BDNF in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. (e) Perinatal arsenic exposure does not alter the protein expression of cAMP responsive element binding protein (CREB) in the nuclear fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of CREB in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. *** $p < .001$, ** $p < .01$, * $p < .05$.