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Prenatal arsenic exposure alters REST/NRSF and microRNA regulators of embryonic neural stem cell fate in a sex-dependent manner

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ABSTRACT

Exposure to arsenic, a common environmental toxin found in drinking water, leads to a host of neurological pathologies. We have previously demonstrated that developmental exposure to a low level of arsenic (50 ppb) alters epigenetic processes that underlie deficits in adult hippocampal neurogenesis leading to aberrant behavior. It is unclear if arsenic impacts the programming and regulation of embryonic neurogenesis during development when exposure occurs. The master negative regulator of neural-lineage, REST/NRSF, controls the precise timing of fate specification and differentiation of neural stem cells (NSCs). Early in development (embryonic day 14), we observed increased expression of *Rest*, its co-repressor, *CoREST*, and the inhibitory RNA binding/splicing protein, *Ptbp1*, and altered expression of mRNA spliced isoforms of *Pbx1* that are directly regulated by these factors in the male brain in response to prenatal 50 ppb arsenic exposure. These increases were concurrent with decreased expression of microRNA-9 (miR-9), miR-9*, and miR-124, all of which are REST/NRSF targets and inversely regulate *Rest* expression to allow for maturation of NSCs. Exposure to arsenic decreased the formation of neuroblasts *in vitro* from NSCs derived from male pup brains. The female response to arsenic was limited to increased expression of *CoREST* and *Ptbp2*, an RNA binding protein that allows for appropriate splicing of genes involved in the progression of neurogenesis. These changes were accompanied by increased neuroblast formation *in vitro* from NSCs derived from female pups. Unexposed male mice express transcriptomic factors to induce differentiation earlier in development compared to unexposed females. Thus, arsenic exposure likely delays differentiation of NSCs in males while potentially inducing precocious differentiation in females early in development. These effects are mitigated by embryonic day 18 of development. Arsenic-induced dysregulation of the regulatory loop formed by REST/NRSF, its target microRNAs, miR-9 and miR-124, and RNA splicing proteins, PTBP1 and 2, leads to aberrant programming of NSC function that is perhaps perpetuated into adulthood inducing deficits in differentiation we have previously observed.

1. Introduction

The transition from quiescent neural stem cells (NSC) to terminally differentiated neurons in the developing brain is highly choreographed *via* a multitude of regulatory factors. These factors mediate the expression levels of neuronal genes to produce stage-specific neurogenesis, including proliferation, fate determination, and differentiation of NSCs into neurons. Developmental exposure to heavy metals reduces NSC proliferation and/or differentiation in the adult brain (Falluel-Morel et al., 2007; Huang et al., 2012; Rai et al., 2010; Senut et al., 2014; Soderstjerna et al., 2013; Taniguchi et al., 2014). We have demonstrated that perinatal exposure of 50 μg/L arsenic (through all three trimesters of development) results in reduced differentiation but not proliferation of NSCs in adult male mice (Tyler and Allan, 2013; Tyler et al., 2014); these findings are corroborated by other studies using *in vivo* and *in vitro* models of arsenic exposure at different time points (Liu and Bain, 2014; Liu et al., 2012). Additionally, arsenic-induced deficits in neuronal differentiation are associated with reduced expression of neural-specific transcription factors in the adult (Hong and Bain, 2012; Tyler and Allan, 2013; Tyler et al., 2015b; Wang et al., 2010), yet the effect of arsenic on the regulation and expression of transcription factors during embryonic neurogenesis has not been studied to date.

NSC fate specification and maturation of neurons are both regulated by multiple epigenetic, transcriptional, and posttranscriptional mechanisms, including splicing and microRNA interactions. Engaging all of these regulatory mechanisms, NRSF, or neuron restrictive silencing factor, also known as the RE-1 silencing transcription factor, REST, induces the silencing of pro-neural genes in multiple cell types (Hsieh and Gage, 2004; Namihira et al., 2008). This zinc finger protein binds to a conserved 21–23 bp motif (RE1) in the promoter regions of its target genes; the NRSF/REST co-repressor, Co-REST, recruits epigenetic

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machinery, including chromatin remodeling complexes, histone modifiers, and DNA binding proteins to alter the epigenetic landscape surrounding the pro-neural gene for silencing. In response to the requirements of the embryonic niche, REST interacts with the microRNAs, miR-9 and miR-124 to control switching from self-renewal of NSCs to differentiation (Packer et al., 2008; Wu and Xie, 2006; Yoo et al., 2009). We have previously demonstrated that our arsenic exposure paradigm alters the expression of chromatin modifications, histone-modifying proteins that associate with REST, and epigenetic regulation of genes with RE-1 motifs, in the adult brain (Tyler et al., 2015a; Tyler et al., 2015b). Whether these epigenetic effects that occur in the adult are induced by altered developmental programming of transcriptional regulation, including REST/NRSF regulatory loops, has not been determined.

In addition to regulation *via* REST, neural progenitors are induced to mature into neurons in part by alternative splicing of RNA transcripts, and appropriate splicing is required for neuronal programming during development (Xue et al., 2013). Polypyrimidine tract-binding proteins (PTBP1 and PTBP2) are widely expressed RNA binding proteins and act to repress alternative RNA splicing, thereby playing a role in post-transcriptional gene regulation (Romanelli et al., 2013). PTB proteins contain RE-1 sites for repression *via* the REST/NRSF complex (Keppetipola et al., 2012). PTBP1 negatively regulates neuron-specific exons in non-neural tissues, while PTBP2 functions as an essential regulator of neuronal maturation in the brain (Tang et al., 2011; Zheng et al., 2012). *In vivo*, *Ptbp1* expression is down regulated by miR-124 resulting in the increased expression of *Ptbp2* and neuronal differentiation (Boutz et al., 2007; Makeyev et al., 2007). Thus, a regulatory loop among REST/NRSF complex, microRNAs and splicing factors controls NSC development.

Here, we expand on our previous work demonstrating that perinatal arsenic exposure alters both the epigenetic landscape and machinery in the adult mouse brain leading to deficits in adult neurogenesis, particularly differentiation. To determine if this altered landscape is due to dysregulated fetal programming, we examined the effect of prenatal exposure to arsenic on the mRNA expression of transcriptional and posttranscriptional modifiers in the brain during the embryonic period. Thus, prenatal arsenic exposure preferentially alters expression of transcriptional modifiers, *Rest* and *CoRest*, and post-transcriptomic modifiers, including RNA binding proteins *Ptbp1*, *Ptbp2*, miR-9, miR-9*, and miR-124, specifically in the male brain early in development and that these effects are not propagated into late development suggesting aberrant *early* programming in the male brain.

2. Materials and methods

2.1. Chemical hazards

Arsenic is classified as a human co-carcinogen; all arsenicals were handled with caution in accordance with MSDS standards.

2.2. Prenatal arsenic exposure paradigm

The Institutional Animal Care and Use Committee at the University of New Mexico (UNM) approved the animal protocols, including the arsenic exposure paradigm, used in this study. C57BL/6 mice obtained from Jackson Labs were maintained on a 12 h reverse light/dark cycle (lights off at 0800) with *ad libitum* access to food and water in the Animal Resource Facility at UNM. Arsenic exposure was performed as previously described (Tyler and Allan, 2013). Briefly, female mice aged 50 days were acclimated to drinking 50 parts per billion arsenic water (sodium arsenate, Sigma Aldrich) for 10–15 days prior to mating. Arsenic water was prepared weekly using 50:50 standard tap and MilliQ water. Control mice were administered tap water from UNM, which contains approximately 2–5 ppb arsenic. Mating occurred on one day; dams continued to drink arsenic-laced water throughout pregnancy until gestational days 14 and 18, when the pups were removed for analysis. Dams were decapitated and embryos removed. Brains were isolated, and the meninges and brain stems were removed. Fetal telencephalonic tissue was dissected for neural progenitor cell (NPC) culture or analyzing mRNA expression. Male and female embryos were identified using qPCR of *Sry* levels in DNA extracted (see below) from the embryo body as described previously. For each experiment, at least seven different litters from seven different dams were used; *e.g.* $n = 7$ represents the number of different litters used with one animal per litter to avoid litter effects.

2.3. Quantitative PCR assessment of mRNA expression

Expression levels of mRNA for *Rest*, *Rcor1* (CoRest), *Ptbp1*, *Ptbp2*, *Pbx1a*, and *Pbx1b* were determined using previously published methods. Briefly, total RNA, including microRNA, was purified with the Ambion mirVana™ miRNA isolation kit (cat: A1561, Life Technologies) following the manufacturer's protocol with slight modification. Telencephalonic tissue homogenate in RLT Plus buffer from the previous gDNA and mRNA co-purification was substituted for tissue lysis with the buffer provided in the mirVana miRNA isolation kit. The standard procedure resumed with the addition of miRNA homogenate additive to the tissue homogenate. Total RNA was quantified either on a NanoDrop 1000 (260/280 ratios > 1.9) or with Life Technology's Broad Range RNA Qubit assay (cat# Q10211) and stored at −80 °C. RNA was treated to remove gDNA and converted to cDNA using Qiagen's QuantiTect Reverse Transcription Kit (cat#205313); cDNA concentration was quantified using Life Technology's ssDNA Qubit assay (cat# Q10212). SYBR green chemistry was used for *Rest*, *Rcor1* (CoRest), *Ptbp1*, and *Ptbp2* assessment; primer information is provided in Table 1. TaqMan chemistry was used for *Pbx1a* and *Pbx1b* assessment; primer/probe sets were as follows: *Pbx1a* (Mm01701536_m1, cat#4331182, Life Technologies), *Pbx1b* (Mm04207622_m1, cat#4331182, Life Technologies), and *Hprt* (Mm00446968_m1, cat#4331182, Life Technologies). In compliance with MIQE standards the following were preformed: melt curves were analyzed to verify a single target was amplified; all qPCR reactions were done in triplicate; no template and reverse transcription negative controls were included on every qPCR plate; and only CT values below 35 cycles were used for analysis (Bustin et al., 2009). Endogenous expression of *Hprt* served as the reference gene for mRNA analysis. We verified that *Hprt* expression itself was not altered by the prenatal arsenic exposure paradigm at both E14 and E18 in both sexes (Supplemental Fig. 1). Gene target fold expression values were obtained using the comparative CT method (Livak and Schmittgen, 2001), and statistical analysis was conducted on GraphPad Prism 6 Software, version 6.03 (GraphPad Software, San Diego, CA).

2.4. Quantitative PCR assessment of microRNA expression

Ten ng of total RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (cat: 4366596; Life Technologies) using primer sets for TaqMan® MicroRNA Assay (cat: 4427975; Life Technologies) for miR-9, miR-9*, miR-18, and miR-124 according to the manufacturer's instructions (Table 1). To produce cDNA suitable for the endogenous control, snoRNA202 (Snord68, cat:4427975; ThermoFisher), RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit and Random Hexamers (50 μM, cat: N8080127; Life Technologies). Previous work identified this RNA as a stable endogenous control for assessments of microRNA expression in our prenatal arsenic exposure paradigm (Caldwell et al., 2015).

2.5. Quantitative PCR determination of sex

Sex determination for embryonic fetal tissue was based on gDNA of the *Sry* gene (sex determining region Y). Validated Sry primer