2016

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Citation/Publisher Attribution

Masoud, A. M., Bihaqi, S. W., Machan, J. T., Zawia, N. H., & Renehan, W. E. (2016). Early-life exposure to lead (Pb) alters the expression of microRNA that target proteins associated with Alzheimer's disease. *Journal of Alzheimer's Disease*, 51(4), 1257-1264. doi: 10.3233/JAD-151018
Available at: http://dx.doi.org/10.3233/JAD-151018

Early-life exposure to lead (Pb) alters the expression of microRNA that target proteins associated with Alzheimer's disease

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Running Title: miRNA response to Pb in a model of AD

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Abstract and Keywords

There is a growing recognition of the impact of environmental toxins on the epigenetic regulation of gene expression, including the genes that play a critical role in neural development, neural function and neurodegeneration. We have shown previously that exposure to the heavy metal lead (Pb) in early life results in a latent over-expression of AD-related proteins in rodents and primates. The present study provides evidence that early postnatal exposure to Pb also alters the expression of select miRNA. Mice were exposed to 0.2% Pb acetate from Postnatal Day 1 (PND 1, first 24 hours after birth) to PND 20 via their mother's milk, brain tissue was harvested at PND 20, 180, or 700 and miRNA were isolated and quantified by qPCR. This exposure produced a transient increase (relative to control) in the expression of miR-106b (binds to *AβPP* mRNA), miR-29b (targets the mRNA for the transcription factor SP1) and two miRNAs (miR-29b and miR-132) that have the ability to inhibit translation of proteins involved in promoter methylation. The expression of miR-106b decreased over time in the Pb-exposed animals and was significantly less than the levels exhibited by the control animals at PND700. The level of miR-124, which binds to *SP1* mRNA, was also reduced (relative to controls) at PND700. In summary, we show that exposure to the heavy metal Pb in early life has a significant impact on the short- and long-term expression of miRNA that target epigenetic mediators and neurotoxic proteins.

Key words: neurodegeneration; miRNA; lead; amyloid-β precursor protein; tau

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and the major cause of dementia. A very small proportion (less than 5%) of individuals with AD develops the disease before age 65, and is considered to have Early Onset Alzheimer's Disease. The overwhelming majority of AD cases are sporadic, or late onset AD (LOAD). To date, no mutation, other than a risk associated with carrying certain susceptibility alleles (currently numbering approximately 24), has been linked to LOAD [1-5]. The failure to identify a clear genetic etiology for LOAD suggests the likelihood that environmental and/or epigenetic factors play an important role in initiating and influencing the cascade of events that leads to this form of AD [6].

There is a growing recognition of the impact of environmental factors such as heavy metals, diet, pesticides and stress on the epigenetic regulation of gene expression, including the genes that play a critical role in neural development, neural function and neurodegeneration [7, 8]. One toxicant that has generated particular concern is the heavy metal lead (Pb), and our laboratory was in fact the first to provide evidence of a potential link between Pb and AD when it demonstrated that exposure to Pb in early life resulted in latent over-expression of AD-related proteins in rodents [9]. We found subsequently that developmental exposure to Pb produced a transient increase in the binding of the transcription factor specificity protein 1 (SP1) to DNA, together with a brief increase in the expression of the mRNA for amyloid-β precursor protein (AβPP) and microtubule-associated protein tau (MAPT) [9-12]. This very transient increase was followed by a delayed increase in SP1 binding together with a delayed overexpression of *AβPP* mRNA, *AβPP*, amyloid-β (Aβ), *Mapt* mRNA and hyperphosphorylated tau in later life [9, 12, 13]. Importantly, these changes do not occur when animals are exposed to Pb in old age. The

results we obtained using our rodent model of environmental Pb exposure have been verified and expanded using tissue obtained from primates that were exposed to Pb during infancy. Tissue obtained from the cerebral cortex of adult (23 years) monkeys exposed to Pb between PND 0-400 exhibited pathologic neurodegeneration, an overexpression of *SP1* mRNA, SP1, AβPP, Aβ, *MAPT* mRNA and tau and a *decrease* in the expression of the methylation-related proteins DNA (Cytosine-5-)-Methyltransferase (DNMT)1, DMNT3a and Methyl CpG Binding Protein 2 (MECP2) [14-16].

Our data regarding Pb-induced changes in SP1, DNMT1, DMNT3a and MeCP2 provide support for the hypothesis that the delayed overexpression of AD-related proteins involves changes to the epigenome. The challenge, therefore, is to identify a mechanism that can explain the delayed emergence of epigenetic modifications that are "triggered" by an environmental stimulus in early life. We suggest that the cancer literature offers some potentially important insights. Work conducted by a number of laboratories has revealed an intriguing interaction between transcription factors, DNMTs and microRNA (miRNA) during oncogenesis [17-22]. The interactions between SP1, the DNMTs and miRNA in oncogenesis provide a framework for developing and testing postulates regarding the roles that epigenetic mediators and transcription factors play in the development of LOAD. Our global hypothesis is that early-life exposure to Pb is a toxic trigger that initiates a cascade of epigenetic changes, beginning with the transient activation of the transcription factor SP1 and its brief activation of select subsets of miRNA. We propose that subsequent accumulating stochastic changes alter miRNA expression and produce a second increase in SP1 expression in later life, resulting in an increase in the transcription of toxic AD-related proteins.

The present study begins the process of testing our global hypothesis by examining the expression of specific miRNA in animals that were exposed to Pb in early life. Recent reviews have identified a small number of miRNA that consistently exhibit increased or decreased expression in AD patients [23, 24]. We used the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/)[25] to select six of these miRNA that target the mRNA for *AβPP, MAPT, DNMT1,3a or 3b, SP1* and/or *MECP2* and quantified their expression in animals that were exposed to Pb to determine whether 1) their expression is altered by Pb exposure and 2) changes in miRNA levels are consistent with their potential role in the delayed overexpression of neurotoxic AD-related proteins.

Materials and methods

Animal exposure

All protocols were conducted in accordance with approval granted by the Institutional Animal Care and Use Committee at the University of Rhode Island (URI). Timed pregnant C57BL/6J strain mice were purchased from Jackson Laboratories (ME, USA) and housed at the Animal Care Facility at URI. Male pups from different dams were pooled and allocated randomly to control and Pb-exposed (PbE) groups. The mothers (N = 9) of the animals in the control group consumed tap water, whereas the mothers (N = 9) of animals in the PbE group drank tap water containing 0.2% Pb acetate from Postnatal Day 1 (PND 1, first 24 hours after birth) to PND 20. Three mice from the control group and three mice from the PbE group were euthanized and their brains harvested at PND 20, 180, or 700 and their hippocampi were stored at -80^{0}C until use (total 9 control and 9 PbE animals). Tissue from these animals was used for the present study as well as our previous investigations that determined the effect of early-life Pb exposure on the expression of *AβPP* mRNA, *AβPP*, Aβ, *Mapt* mRNA and MAPT [12, 26].

Total RNA isolation and miRNA real-time polymerase chain reaction

Total RNA was extracted from control and PbE brain tissue according to the TRIzol method (Invitrogen, CA) as per manufacturer protocol. The quality of extracted RNA was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, NY). Complementary DNA (cDNA) of miRNA was synthesized from 1 µg of total RNA using the NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen, CA) as per manufacturer protocol. The qPCR analyses of synthesized cDNA were performed using SYBR Green qRT-PCR assays (20 µl reactions in replicates, using AβAppropriate amount of cDNA template, 10 µl SYBR

Green master mix, 500 nM final concentration of forward and reverse primers, and deionized water) with amplification performed in the ViiA 7 Real-Time PCR system (Applied Biosystem, CA). The expression was reported relative to sno202 miRNA with the $2^{-\Delta Ct}$ method.

Statistical analysis

Traditionally, real-time quantitative RT-PCR (qPCR) cycles-to-threshold (CT) values are converted to normalized "fold" differences between groups before being subjected to statistical analysis. A difficulty with this method is that CT values are generally Gaussian, whereas fold differences imply proportional effects and variances that change with means. We employed a general linear model (GLM) analytic technique [27, 28] that allowed us to test an array of linear hypotheses accompanying our multi-factored design, including higher-order interactions. All analyses were carried out with SAS version 9.4 (The SAS Institute, Cary, NC). *Proc glimmix* was used to construct general linear models of CT values directly, which mirrored the experimental design. The GLMs accounted for the repeated measures within mouse (multiple miRNAs) using correlated residual errors. This was necessary as the corollary of the within-animal subtraction of the housekeeping gene CT for normalization. A compound symmetry variance-covariance structure was used to fit the model initially. This assumes homogenous variances of miRNA and a common covariance between pairs of miRNA within animal. Classical sandwich estimation was used to automatically adjust for differences in miRNA variance and changes in covariance between pairs of miRNA. The models were 3-way factorial models, with main effects for miRNA, treatment, and age, along with all 2-way and 3-way interactions included in the main model. Individual *a priori* specified hypothesis tests were carried out within the model as complex orthogonal comparisons, comparing groups while normalizing to the housekeeping gene (difference of the difference = ΔΔCT). Family-wise alpha

was maintained at 0.05 using the Holm test, though unadjusted p-values were also presented in some instances. The (geometric) mean and 95%CI of normalized concentrations of target miRNA ($2^{-\Delta CT}$) were used to illustrate how different conditions varied across time.

Results

The expression of miR-106b (targets *AβPP* mRNA), miR-34c (*MAPT* mRNA), miR-124 (*SP1* mRNA), miR-29b (*DNMT3a,b*; *Dnmt3a,b* and *SP1* mRNA), miR-148a (*DNMT1* and *Dnmt1* mRNA) and miR-132 (*MECP2* and *Mecp2* mRNA) was measured using qPCR at PND 20, 180 and 700 in animals that were exposed to Pb (and their controls) from PND 1-20 via mothers' milk.

miR-106b

Figure 1A illustrates the expression of miR-106b, a miRNA that has been shown to target the mRNA for the human *AβPP* gene [29]. The data demonstrate that the animals responded to Pb exposure with an increase in the production of miR-106b, but this increase was not maintained throughout the lifespan. We found that the animals exposed to Pb had a 1.5-fold increase in the expression of miR-106b, relative to control, at PND20 (95% confidence interval 1.1-2.1, p=0.02, adjusted p=0.28). The expression of miR-106b decreased substantially in the Pb-exposed animals between PND20-180 and was 1.7-fold lower than levels seen in age-matched control animals at PND700 (95% CL 2.2-1.1, p=0.0078, adjusted p=0.11). There was no significant change over time in the expression of miR-106b in the animals that were not exposed to Pb.

miR-34c

The expression of miR-34c, a miRNA that targets the mRNA for *MAPT* [30], is shown in Figure 1B. We found that the expression of this miRNA remained relatively unchanged, across the lifespan, in the animals that had been exposed to Pb. Interestingly, this contrasts with the

expression of miR-34c in *normal* animals, which exhibited a dramatic *increase* in miR-34c levels between PND20 and 180. The difference between the two groups was most apparent at PND180, where the expression of miR-34c in the control animals was 1.6-fold greater than that seen in the Pb-exposed animals (95% CI 2.0-1.4, $p<0.0001$, adjusted $p<0.0001$).

miR-124

Figure 1C illustrates the expression of miR-124, a miRNA that targets the mRNA for *SP1* [31]. The levels of miR-124 in the Pb-exposed animals remained relatively unchanged between PND20 and 180 and reflected the levels seen in control animals. This profile changed dramatically, however, between PND180-700. The expression of miR-124 in the animals exposed to Pb decreased dramatically in this interval, and at PND700 the level of miR-124 in the Pb-exposed animals was *2.0-fold less* than the controls (95% CI 3.8-1.1, $p=0.02$, adjusted $p=0.33$). There was no significant change over time in the expression of miR-124 in the animals that were not exposed to Pb.

miR-29b

The expression of miR-29b, a miRNA that targets the mRNA for *DNMT3* [32], *Dnmt3* [33] and *SP1* [34-37] mRNA, is illustrated in Figure 1D. The level of miR-29b in the animals exposed to Pb was 1.6-fold greater than the level exhibited by controls on PND20 (95% CI 1.4-1.9, $p<0.0001$, adjusted $p<0.0001$). Both groups exhibited a significant increase in the expression of this miRNA between PND20 and PND180, with the increase associated with the control group particularly striking. The expression of miR-29b did not change for either group between PND180-700.

miR-148a

Figure 1E depicts the expression of miR-148a, which has documented targets on *DNMT1* [38] and *Dnmt1* [39] mRNA. Comparisons at each of the three time points failed to identify any significant differences in the expression of miR-148a in Pb-exposed and control animals.

miR-132

The expression of miR-132, which targets *MECP2* [40] and *Mecp2* [41] mRNA, is illustrated in Figure 1F. We found that early life Pb exposure produced a dramatic increase (relative to normal) in the expression of this miRNA at PND20. The level of miR-132 in the Pb-exposed animals was 4.8-fold greater than the level associated with the control animals (95% CI 2.4-9.7, $p<0.0001$, adjusted $p=0.0004$).

Discussion

The present study demonstrates that exposure to the heavy metal Pb in early life has a significant impact on the expression of miRNA that target epigenetic mediators and neurotoxic proteins. Brief developmental exposure to this toxicant produced an early postnatal increase (relative to control) in the expression of a miRNA (miR-106b) that has been shown to bind to the 3' untranslated region of *AβPP* mRNA, a miRNA (miR-29b) that targets *SP1* and two miRNAs (miR-29b and miR-132) that have the ability to inhibit translation of proteins involved in promoter methylation. The expression of miR-106b decreased over time in the Pb-exposed animals and was significantly less than the levels exhibited by the control animals at PND700. The level of miR-124, which binds to *SP1* mRNA, was also reduced (relative to controls) at PND700.

How might we understand the evolution and potential impact of these changes in miRNA expression? In previous studies, using the same animals employed in the present investigation, we have demonstrated that developmental exposure to Pb produced a transient increase in the binding of SP1 to DNA, together with a brief increase in the expression of *AβPP* and *Mapt* mRNA [9-12]. This brief increase in *AβPP* and *Mapt* mRNA was followed by an extended "latent" period during which the levels of these mRNA were indistinguishable from normal. As the animals approach PND700, however, there was a latent overexpression of *AβPP* mRNA, *AβPP*, Aβ, *Mapt* mRNA and hyperphosphorylated tau [9, 12, 13]. We propose that the Pb-induced transient increase in SP1 binding to DNA allows the transcription factor to increase transcription of select miRNA, allowing them to participate in the normalization of AβPP, SP1 and MAPT levels that lasts for many months in rodents and many years in primates [9, 11, 14, 42-45]. Increased expression of miR-29b in the early postnatal period, for example, would

conceivably allow this miRNA to inhibit the production of SP1, while the early postnatal increase in miR-106b would inhibit the expression of AβPP. Meanwhile, increased production of miR-29b and miR-132 would act to inhibit the translation of the methylation-related proteins. This decrease in the expression of the DNMTs and MECP2 would facilitate the continued expression of miR-106b and miR-29b if their expression is sensitive to promoter methylation.

We predict that the miRNA that participate in the regulation of AD-related proteins are subject to accumulating stochastic changes similar to those associated with miRNA that play a role in oncogenesis [46-48]. We propose that the symptomatic period of LOAD is initiated by a reduction in the expression of some of the miRNA targeting *SP1, AβPP*, and *MAPT* mRNA, producing a decrease in post-transcriptional inhibition of *SP1, AβPP* and *MAPT*. *This would result in an increase in the expression of neurotoxic Aβ and tau.* Though the increased availability of SP1 and continued hypomethylation of the miR-106b and miR-124 promoters would seem to afford an opportunity to enhance transcription of these miRNA, we suggest that the loss-of-function changes to these miRNA will have greater influence.

Conclusion

We have shown that exposure to the heavy metal Pb in early life has a significant impact on the expression of miRNA that target epigenetic mediators and neurotoxic proteins. Three of the six miRNA we studied exhibited an *increase in expression* in the period immediately following the Pb exposure and we suggest that these and similar miRNA may participate in the post-exposure normalization of gene expression that we have described previously. The expression of two miRNA *decreased* over time in the Pb-exposed animals and we predict that

these and related decreases in specific cerebral miRNA contribute to the over-expression of neurotoxic proteins in later life.

Acknowledgements

This research was supported by NRF Fund No. 31M091 and the National Institute of Environmental Health Sciences grant NIH ES015867. The research was made possible by the use of the RI-INBRE Research Core Facility, supported by the NCRR/NIH Grant #P20 RR016457.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations used: Aβ: Amyloid-beta; AD: Alzheimer's disease; AβPP: Amyloid precursor protein; DNMT: DNA methyltransferase; LOAD: late onset Alzheimer's disease; MAPT: Microtubule-associated protein tau; MeCP2: Methyl CpG binding protein 2; miRNA: microRNA; NFT: Neurofibrillary tangle; PND: Postnatal day; SP1: Specificity protein

Table 1

Target	miRBase ID	Primer sequence
miR-29b	MI0000143	5'-GCTGGTTTCATATGGTGGTTTA-3'
miR-34c	MI0000403	5'-AGGCAGTGTAGTTAGCTGATTGC-3'
miR-106b	MI0000407	5'-TAAAGTGCTGACAGTGCAGAT-3'
miR-124	MI0000716	5'-CGTGTTCACAGCGGACCTTGAT-3'
miR-132	MI0000158	5'-AACCGTGGCTTTCGATTGTTAC-3'
miR-148	MI0000550	5'-AAAGTTCTGAGACACTCCGACT-3'
sno-202		Forward: 5'-GCTGTACTGACTTGATGAAG-3' Reverse: 5'-CATCAGATGGAAAAGGGTTC-3'

Table 1. miRNA and housekeeping gene primers

Fig. 1 Expression of miRNA relative to sno202 in control animals (dotted line) and Pb-exposed animals (PbE, solid line) at PND 20, 180 and 700. Results expressed as mean ± 95% CI using the Holm post hoc test ($p<0.05$ considered significant compared to control, indicated by *), n=3 with measurements in triplicates.

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