Published in final edited form as:

J Alzheimers Dis. 2018 ; 63(1): 273–282. doi:10.3233/JAD-170824.

Altered microRNA, mRNA, and Protein Expression of Neurodegeneration-Related Biomarkers and Their Transcriptional and Epigenetic Modifiers in a Human Tau Transgenic Mouse Model in Response to Developmental Lead Exposure

Anwar M. Masoud^d, Syed W. Bihaqi^c, Bothaina Alansi^a, Miriam Dash^b, Gehad M. Subaiea^e, William E. Renehan^c, Nasser H. Zawia^{a,b,c,*}

^aDepartment of Biomedical and Pharmaceutical Science, University of Rhode Island, Kingston RI, USA

^bInterdisciplinary Neuroscience Program, University of Rhode Island, Kingston RI, USA

^cGeorge and Anne Ryan Institute for Neuroscience, University of Rhode Island, Kingston RI, USA

^dBiochemical Technology Program, Faculty of Applied Science, Thamar University, Thamar, Yemen

^eDepartment of Pharmacology and Toxicology, College of Pharmacy, University of Hail, Hail, Kingdom of Saudi Arabia

Abstract

Amyloid deposits originating from the amyloid- β protein precursor (A β PP) and aggregates of the microtubule associated protein tau (MAPT) are the hallmarks of Alzheimer's disease (AD). Animal studies have demonstrated a link between early life exposure to lead (Pb) and latent overexpression of the A β PP and MAPT genes and their products via epigenetic reprogramming. The present study monitored *APP* gene and epigenetic mediators and transcription factors known to regulate it. Western blot analysis and quantitative polymerase chain reaction (qPCR) were used to study the mRNA, miRNA, and proteins levels of A β PP, specificity protein 1 (SP1; a transcriptional regulator of amyloid and tau pathway), and epigenetic intermediates namely: DNA methyltransferase (DNMT) 1, DNMT3a and Methyl-CpG protein binding 2 (MeCP2) in the cerebral cortex of transgenic mice (Knock-in for human MAPT). These transgenic mice were developmentally exposed to Pb and the impact on mRNA, miRNA, and protein levels was scrutinized on postnatal days (PND) 20 and 50. The data revealed a consistent inverse relationship between miRNA and protein levels for SP1 and A β PP both in the basal and exposed conditions, which may influence the levels of their corresponding proteins. On the other hand, the relationship between miRNA and protein levels was not correlative for DNMT1 and DNMT3a. MeCP2 miRNA protein levels corresponded only following environmental exposure. These results suggest

*Correspondence to: Nasser H. Zawia, PhD, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, 02881, USA. Tel.: +1 401 874 2663; Fax: +1 401 874 5491; nzawia@uri.edu.

Authors' disclosures available online (<https://www.j-alz.com/manuscript-disclosures/17-0824r3>).

that developmental exposure to Pb and subsequent A β PP protein levels may be controlled through transcriptional regulators and epigenetic mechanisms that mainly involve miRNA regulation.

Keywords

Alzheimer's disease; A β PP; DNMT3a; lead; MAPT; miRNA; SP1

INTRODUCTION

Alzheimer's disease (AD) is the major cause of dementia, currently affecting one in ten Americans over 65 years of age and one in three people 85 years and older [1]. The majority of AD cases (more than 95%) are sporadic known as late onset AD or LOAD [2]. There is a growing awareness that environmental and/or epigenetic factors may play an important role in initiating and influencing the cascade of events that leads to LOAD [3].

We provided the first evidence that early life exposure to lead (Pb) can be a risk factor that promotes the pathogenesis of AD. Previous reports from our lab have shown that expression of AD-related genes as well as their transcriptional regulator, specificity protein 1 (SP1) are altered in rodents and primates exposed to Pb as infants [4–7]. These characteristics are manifested by an increase in the expression of the proteins associated with amyloidogenesis and tauopathy, as well as behavioral deficits in aged rodents with prior exposure to Pb [4, 8–10]. Additional reports from our laboratory (using mouse and primate models) have shown that changes in the expression of AD related genes are driven by factors involved in epigenetic regulation pathways, such as DNA methyltransferases (DNMTs) and histone modifiers [7, 11]. Recently, we reported a role for microRNA (miRNA) in mediating exposure-related changes in gene expression in wild-type mice [12].

While the above studies involved only wild type rodents or primates, our laboratory is currently pursuing a series of investigations that seek to determine whether the human tau (hTau) gene is also sensitive to early-life exposure to environmental toxins. We have demonstrated that exposure to Pb in the early postnatal period (PND1–20) produces a transient increase in the expression of hTau and phosphorylated hTau (at Ser-396) at PND 20 and 30, followed by a normalization of tau levels. Interestingly, the decrease in tau was coincident with an increase in the expression of miR-34c; a micro-RNA (miRNA) that is known to target *MAPT* mRNA [13]. These data thus indicate that miRNAs may play a role in regulating the expression of the human *MAPT* gene and also suggests of the possible involvement of miRNAs in regulating the expression of other key AD-related genes, such as *APP*.

The present study was designed to investigate whether miRNA might be involved in regulation of the murine *APP* gene, its transcriptional regulator SP1 and other epigenetic mediators that impact the expression of this gene. We used the miRTarBase database (<http://mirtarbase.mbc.nctu.edu.tw/>) [14] to select five of these miRNA: 1) miR-29b (targets *DNMT3a* mRNA, 2) miR-124 (targets mRNA of *SP1*), 3) miR-132 (known to target *MeCP2* mRNA), 4) miR-148 (targets mRNA of *DNMT1*), and 5) miR-106b that binds to *APP* mRNA [15–24]. The miRNA, and their corresponding mRNA, and protein products were

monitored to reveal whether changes in miRNA had any correlative relationship that might influence the final outcome of AD-related biomarker levels.

MATERIAL AND METHODS

Animals and exposure

A transgenic mouse model B6.Cg-Mapt^{tm1 (GFP)KltTg(MAPT)8cPdav/J} (Jackson Laboratory Stock 005491, Bar Harbor, ME) that expresses the six tau isoforms (including both 3R and 4R forms) of human MAPT was used in the present study. Mice were bred in the Animal Care Facility at the University of Rhode Island (Kingston, RI). Using software from <http://statpages.org/#power> generated a sample size of 8 animals per group with the parameters set at 40% anticipated difference in means, 25% anticipated standard deviation of the mean values, 0.8 power and 0.05 type I error. Two additional animals will be included in each group to account for any unexpected problems. Postnatal day 1 (PND 1) was designated as 24 h after birth. All pups were pooled, and new litters consisting of 8 males were selected randomly and placed with each dam. The mice were divided into two groups. Group 1: served as control and received tap water. Group 2: (Pb-E, the early Pb exposure group) was exposed to 0.2% Pb acetate from PND 1 to PND 20 through drinking water of the dam. [10, 13].

Pups were euthanized by CO₂ inhalation at PND20 and PND50 for mRNA, miRNA, and protein levels assays (N = 5 each group and time point) and the brains were removed and placed on ice followed by immediate storage at -80°C. The study was performed in accordance with the standard guidelines and the protocol was approved by the University of Rhode Island Institutional Animal Care and Use Committee (IACUC).

Brain Pb concentration

Elemental profiling via inductively coupled plasma mass spectrometry (ICP-MS) was performed for Pb in cortical brain tissue. Samples were prepared by concentrated HNO₃ and diluted in Milli-Q water to a final concentration of 2% HNO₃. ICP-MS was performed using a ICAP-Q (Thermo-Fisher Scientific, MA, USA). The samples were introduced with a segmented flow technique (1.0 ml/min; 14 s for each sample) through a Meinhard nebulizer and a chilled spray chamber. No flow injection valve was used; the auto-sampler was simply programmed to stay in the sampling position for the specified time. The argon plasma conditions were: forward power 1350W; reflected power <5W; nebulizer gas flow rate 0.9, intermediate and outer gas flow rates 0.8 and 13.0 l/min, respectively. The instrument was calibrated using 2% HNO₃ solution containing Pb at 100, 200, and 300 parts per billion (ppb).

Protein extraction and western blot analysis

Cerebral cortices from both hemispheres were randomly used and homogenized in radioimmunoprecipitation assay lysis buffer RIPA, containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 2 µL protease inhibitor, and 10 µL phosphatase inhibitor), incubated in ice for 1 h. The supernatants obtained after centrifugation of homogenates at

8,000 rpm for 10 min at 4°C were collected and used for western blot analysis. Pierce bicinchoninic assay (BCA) kit (Thermo Scientific, Waltham, MA, USA) was used to determine the protein concentration. Amounts of 20–40 µg of total protein were separated by electrophoresis onto 10–12% polyacrylamide gel at 100 V for 2 h and then transferred to polyvinylidene fluoride (PVDF) membranes (GE-Healthcare, Piscataway, NJ, USA). The nonspecific bindings were blocked by incubation with 5% bovine serum albumin (BSA) in Tris-buffer Saline +0.1% Tween 20 (TBST) at room temperature for 1 h. Following overnight incubation, the immunoblotting was performed using antibodies diluted at 1:1000 with gentle agitation on a shaker at 4°C. The used antibodies were as follow: AβPP (MAB 348: Anti-AβPP A4 Antibody, a.a. 66–81 of AβPP [N-terminus], clone 22C11, Millipore, MA, USA); MeCP2 (D4F3: XP® Rabbit mAb, Cell Signaling Tech, Danvers, MA, USA); DNMT1 (D63A6: XP® Rabbit mAb, Cell Signaling Tech., Danvers, MA, USA); DNMT3a (D23G1: Rabbit mAb, Cell Signaling Tech., Danvers MA, USA); and SP1 (07–645: diluted at 1:500, Milli-pore, MA, USA). The membranes were washed and exposed for 1 h to goat anti-mouse/goat anti-rabbit IRDye® 680LT Infrared Dye diluted at 1:10,000 (LI-COR Biotechnology, Lincoln, NE, USA). The images were developed using an Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA). Membranes were re-probed for GAPDH (Anti-GAPDH antibody produced in rabbit, Sigma Aldrich, MO, USA) to obtain protein/GAPDH ratio. The intensities of the Western blot bands were measured using Odyssey V1.2 Software (LI-COR Biotechnology, Lincoln, NE, USA).

Total RNA isolation, synthesis of complementary DNA, and quantitative polymerase chain reaction (q-PCR)

RNA from the brain tissue was isolated according to the TRIzol method (Invitrogen, Carlsbad, CA, USA). The quality of extracted RNA was verified using a Nano-Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) was synthesized from 1 µg total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) for mRNA, and NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) for miRNA as per manufacturer protocol. The qPCR analyses of synthesized cDNA were performed using SYBR Green quantitative real-time PCR assay reactions (12.5 µL for mRNA or 20 µL for miRNA), 1 µL cDNA template, 1X SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 0.5 µM forward and reverse primers, and deionized H₂O. Amplification was carried in ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the standard protocol: 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were analyzed relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for mRNA and relative to small nucleolar RNA 202 (sno202) for miRNA with the 2^{-CT} method [25].

Statistical analysis

All measurements were performed in triplicate for N = 3 of each group and all values are presented as mean ± standard error of the mean using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple-comparison post hoc test. The level of significance for each ANOVA was set at $\alpha = 0.05$. All analyses were performed using Sigma

Stat 3.5 computer software (San Jose, CA, USA). In addition, Pearson correlation analysis was conducted to measure the strength of a linear association between two variables.

RESULTS

The levels of miRNA and their mRNA targets (miR-29b targets DNMT3a mRNA, miR-106b targets *APP* mRNA, miR-124 targets *SP1* mRNA, miR-132 targets *MeCP2* mRNA and miR-148a targets *DNMT1* mRNA) were measured using real-time quantitative RT-PCR (qPCR) whereas; the corresponding proteins were measured by Western blot analysis at PND20 and PND50. The cycles-to-threshold (CT) values for each mRNA and miRNA were normalized to the CT values obtained for *GAPDH* and sno202 (reference/housekeeping gene), respectively.

Brain Pb levels in hTau mice

Brain samples from transgenic control and developmentally Pb exposed mice were subjected to quantitative ICP-MS measurements of Pb. On PND20, a mean Pb concentration of 0.27 ppm of wet weight was detected in unexposed mice, while an average of 1.95 ppm of wet weight was measured in Pb-exposed mice. On PND50, the mean Pb concentration was 0.015 ppm in unexposed mice, and 0.77 ppm in Pb exposed ones.

MiR-106b and its target APP mRNA and protein levels

Figure 1a and 1b illustrates the changes in the pattern of *APP* mRNA and miR-106b. *APP* mRNA of PbE was significantly ($p < 0.05$) low at PND20 as compared to control animals, and recovered to that of the controls at PND50 (Fig. 1a, $p = 0.014$). MiR-106b followed the same pattern as the *APP* mRNA in both control and exposed animals (Fig. 1b, $p = 0.004$). Importantly, the high level of miR-106b at PND50 in the Pb-exposed animals correlated well with a significant ($p < 0.05$) reduction in the A β PP protein level (Fig. 1c and 1d). There was a consistent inverse relationship between miR-106b and A β PP protein levels in control and exposed animals (Fig. 1e). Our results further demonstrated that correlation exists between C20 of miR-106b and Pb-E 20 for A β PP ($r = -0.9988$), furthermore correlation was also observed between Pb-E 20 and Pb-E 50 for A β PP ($r = +0.9978$).

MiR-124 and its target SP1 mRNA and protein levels

The expression of *SP1* mRNA in Pb-exposed animals at PND20 was greater than that measured in controls ($p = 0.018$, Fig. 2a). The levels of *SP1* mRNA increased in both control and Pb-exposed animals at PND20 and PND 50. Meanwhile, expression of miR-124 showed a significant increase at PND50 in PbE animals compared with control animals (Fig. 2b, $p = 0.026$). SP1 protein levels exhibited a consistent inverse relationship relative to the levels of miR-124, while the changes in mRNA did not match that of the protein levels (Fig. 2c, d). In summary, good correspondence was seen between miRNA and protein levels (Fig. 2e). Pearson correlation analysis showed that there was a positive correlation in those genes where we found a relationship between the levels of miRNA and protein at both the basal and exposed conditions. For SP1 the r value was 0.99 or in the exposure scenario at PND 50.

MiR-148a and its target DNMT1 mRNA and protein levels

A significant high level of *DNMT1* mRNA at PND50 was observed in the control animals compared with Pb-E (Fig. 3a, $p = 0.036$), while this pattern remained relatively flat following Pb-exposure. The expression of miR-148a was lower at PND50 compared to PND20 in control animals, while the expression of miR-148a in Pb-E animals was high at PND50 (Fig. 3b, $p = 0.018$) as compared to aged matched control animals. Figure 3c and 3d showed that the levels of DNMT1 protein increased in both control and Pb-E animals at PND20 and PND 50. While miR-148a expression was inversely related to that of the DNMT1 protein in control animals, this was not the case in the animals exposed to Pb (Fig. 3e).

MiR-29b and its target DNMT3a mRNA and protein levels

The expression of *Dnmt3a* mRNA at PND20 and PND50 was higher in Pb-E animals as compared to control animals, but achieved significance only at PND50 ($p = 0.048$, Fig. 4a). Likewise, the level of miR-29b was significantly higher in the Pb-exposed animals at PND50 ($p = 0.015$, Fig. 4b). There was, however, no evidence that miR-29b was impacting the expression of the DNMT3a protein (Fig. 4e).

MiR-132 and its target MeCP2 mRNA and protein levels

There was no significant difference in the expression of *MeCP2* mRNA or miR-132 in control and Pb-E animals at either PND20 or PND50 (Fig. 5a, b). The expression of MECP2 protein, however, was significantly reduced (compared to control) in the Pb-E animals (Fig. 5c, d). Thus, there was good correspondence between miRNA and protein levels in Pb-exposed animals only (Fig. 5e).

DISCUSSION

Mature miRNAs are small RNA nucleotides (19–25 nucleotides) that bind to mRNA and play an important role in the development and function of the brain [26]. They repress translation or degrade mRNA transcripts by binding to the 3'UTR region of mRNA [27, 28]. Many researchers have reported dysregulation of miRNAs in AD resulting in overexpression of AD-related proteins [29–32]. It has also been suggested that miRNAs play an important role in Pb toxicity in the hippocampus of rodents [33]. We recently reported that developmental exposure to Pb in human tau mice results in a transient increase in levels of hTau, its phosphorylated site Ser396 and tau mRNA. In that same study, we also observed that, miR-34c which targets MAPT was over-expressed at PND50, coincident with a decrease in the levels of both tau and its mRNA [13]. mRNA and miRNA expression is expected to change during development [34]. The findings reinforced the miRNA pathway as a predominant mechanism via which developmental exposure to Pb influences latent expression of AD-related biomarkers.

Here, we investigated the alterations in the expression of the AD-related gene (*APP*) and its epigenetic and transcriptional regulators *DNMT1*, *DNMT3a*, *MeCP2*, and *SP1* following exposure to Pb. Previous work had looked at the AD-related gene tau [12]. Here we found that Pb exposure elevated the background levels of Pb in the tissues of these mice almost

five-fold after 20 days of exposure. Once exposure ended at PND50, the levels of Pb in the tissues dropped back to about 0.77 ppm. The latent effects on biomarkers occurred at PND50 after the Pb concentrations have returned to near background levels.

Variable results were obtained when comparing mRNA, miRNA, and protein levels for each biomarker. The best inverse relationship was observed between miRNA and protein levels for each biomarker following Pb exposure, and the most consistent changes in biomarker levels at both the control and exposed condition were observed for SP1 and A β PP. Pearson correlation analysis showed that there was a positive correlation ($r = 0.99$) in those genes in which we found a relationship between the levels of miRNA and protein at both the basal and exposed conditions. Given that multiple miRNAs may target one gene and that one miRNA may target multiple genes, these results suggest an important role for miRNA regulation and its impact on transcriptional control and epigenetic pathways.

SP1, the zinc finger transcription factor, regulates the expression of A β PP and tau, and its elevation has been reported in AD patients and transgenic animal models of AD [4, 5, 35–37]. We had previously reported elevated SP1 protein and mRNA in response to Pb exposure in rodents and primates [8, 9, 38] and postulated that this elevation was responsible for alterations in the levels of AD-related proteins in the hippocampus of mice exposed to Pb in early life [12]. In the present study, we have seen a significant high level in the *SP1* mRNA at PND20 following exposure to Pb with a significant increase in the SP1 protein at the same time point consistent with its role as a regulator of AD-related genes. Recently, it has been suggested that miR-124-3p targeting *SP1* mRNA plays a protective role through attenuating the hyperphosphorylation of tau-induced cell apoptosis in AD [39]. These findings reinforce the role of SP1 in regulating genes associated in neurodegeneration.

Epigenetic modifications include DNA methylation and binding proteins such as DNMT1, DNMT3a, and MeCP2. We observed that miRNA played a minor role in controlling the levels of DNMT1 and DNMT3a. On the other hand, miRNA impacted MeCP2 only following Pb exposure. While the *APP* gene is highly CpG rich, control of A β PP protein levels appears to be a function of miRNA regulation rather than a product of methylation control at the gene level. This is consistent with our past reports that performed genome-wide methylation and mRNA expression studies, in which we reported that early-life exposure to Pb results in a latent widespread repression of the genome in old age [40, 41]. These genome wide studies were inconsistent with *APP* mRNA and protein levels which were upregulated late in life.

Conclusion

Variable results were obtained when comparing mRNA, miRNA, and protein levels for each biomarker. The best inverse relationship was observed between miRNA and protein levels for each biomarker following Pb exposure, and the most consistent changes across the biomarkers were observed for SP1 and A β PP. It is important to note that protein degradation and turn-over was not measured in these studies and these initial observations have focused on the direct pathway from DNA to protein. While this study is descriptive in nature and requires more direct evidence to show that the miRNAs are interfering with the translation of the message into protein, these results suggest that developmental exposure to Pb impacts

subsequent protein levels of AD biomarkers through a pathway that mainly involves miRNA regulation.

ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health (NIH) and by the NIH grants ES13022, AG027246, 1R56ES01568670-01A1, and 1R01ES015867-01A2 awarded to NHZ. The research was made possible by the use of the RI-INBRE Research Core Facility, supported by the NIGMS/NIH Grant # P20GM103430.

REFERENCES

- [1]. Alzheimer's Association (2015) 2015 Alzheimer's disease facts and figures. *Alzheimers Dement* 11, 332–384. [PubMed: 25984581]
- [2]. Rosenthal SL, Kamboh MI (2014) Late-onset Alzheimer's disease genes and the potentially implicated pathways. *Curr Genet Med Rep* 2, 85–101. [PubMed: 24829845]
- [3]. Lunnon K, Mill J (2013) Epigenetic studies in Alzheimer's disease: Current findings, caveats, and considerations for future studies. *Am J Med Genet B Neuropsychiatr Genet* 162b, 789–799. [PubMed: 24038819]
- [4]. Basha MR, Wei W, Bakheet SA, Benitez N, Siddiqi HK, Ge YW, Lahiri DK, Zawia NH (2005) The fetal basis of amyloidogenesis: Exposure to lead and latent overexpression of amyloid precursor protein and beta-amyloid in the aging brain. *J Neurosci* 25, 823–829. [PubMed: 15673661]
- [5]. Wu J, Basha MR, Brock B, Cox DP, Cardozo-Pelaez F, McPherson CA, Harry J, Rice DC, Maloney B, Chen D, Lahiri DK, Zawia NH (2008) Alzheimer's disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): Evidence for a developmental origin and environmental link for AD. *J Neurosci* 28, 3–9. [PubMed: 18171917]
- [6]. Bihaqi SW, Zawia NH (2013) Enhanced tauopathy and AD-like pathology in aged primate brains decades after infantile exposure to lead (Pb). *Neurotoxicology* 39, 95–101. [PubMed: 23973560]
- [7]. Bihaqi SW, Huang H, Wu J, Zawia NH (2011) Infant exposure to lead (Pb) and epigenetic modifications in the aging primate brain: Implications for Alzheimer's disease. *J Alzheimers Dis* 27, 819–833. [PubMed: 21891863]
- [8]. Zawia NH, Sharan R, Brydie M, Oyama T, Crumpton T (1998) Sp1 as a target site for metal-induced perturbations of transcriptional regulation of developmental brain gene expression. *Brain Res Dev Brain Res* 107, 291–298. [PubMed: 9593950]
- [9]. Atkins DS, Basha MR, Zawia NH (2003) Intracellular signaling pathways involved in mediating the effects of lead on the transcription factor Sp1. *Int J Dev Neurosci* 21, 235–244. [PubMed: 12850056]
- [10]. Bihaqi SW, Bahmani A, Adem A, Zawia NH (2014) Infantile postnatal exposure to lead (Pb) enhances tau expression in the cerebral cortex of aged mice: Relevance to AD. *Neurotoxicology* 44, 114–120. [PubMed: 24954411]
- [11]. Eid A, Bihaqi SW, Renehan WE, Zawia NH (2016) Developmental lead exposure and lifespan alterations in epigenetic regulators and their correspondence to biomarkers of Alzheimer's disease. *Alzheimers Dement (Amst)* 2, 123–131.
- [12]. Masoud AM, Bihaqi SW, Machan JT, Zawia NH, Renehan WE (2016) Early-life exposure to lead (Pb) alters the expression of microRNA that target proteins associated with Alzheimer's disease. *J Alzheimers Dis* 51, 1257–1264. [PubMed: 26923026]
- [13]. Dash M, Eid A, Subaiea G, Chang J, Deeb R, Masoud A, Renehan WE, Adem A, Zawia NH (2016) Developmental exposure to lead (Pb) alters the expression of the human tau gene and its products in a transgenic animal model. *Neurotoxicology* 55, 154–159. [PubMed: 27293183]
- [14]. Hsu SD, Tseng YT, Shrestha S, Lin YL, Khaleel A, Chou CH, Chu CF, Huang HY, Lin CM, Ho SY, Jian TY, Lin FM, Chang TH, Weng SL, Liao KW, Liao IE, Liu CC, Huang HD (2014) miRTarBase update 2014: An information resource for experimentally validated miRNA-target interactions. *Nucleic Acids Res* 42, D78–85. [PubMed: 24304892]

- [15]. Hebert SS, Horre K, Nicolai L, Bergmans B, Papadopoulou AS, Delacourte A, De Strooper B (2009) MicroRNA regulation of Alzheimer's Amyloid precursor protein expression. *Neurobiol Dis* 33, 422–428. [PubMed: 19110058]
- [16]. Klein ME, Liroy DT, Ma L, Impey S, Mandel G, Goodman RH (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. *Nat Neurosci* 10, 1513–1514. [PubMed: 17994015]
- [17]. Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* 104, 15805–15810. [PubMed: 17890317]
- [18]. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* 113, 6411–6418. [PubMed: 19211935]
- [19]. Jia LF, Huang YP, Zheng YF, Lyu MY, Wei SB, Meng Z, Gan YH (2014) miR-29b suppresses proliferation, migration, and invasion of tongue squamous cell carcinoma through PTEN-AKT signaling pathway by targeting Sp1. *Oral Oncol* 50, 1062–1071. [PubMed: 25127200]
- [20]. Li J, Du S, Sheng X, Liu J, Cen B, Huang F, He Y (2016) MicroRNA-29b inhibits endometrial fibrosis by regulating the Sp1-TGF-beta1/Smad-CTGF axis in a rat model. *Reprod Sci* 23, 386–394. [PubMed: 26392347]
- [21]. Villa C, Ridolfi E, Fenoglio C, Ghezzi L, Vimercati R, Clerici F, Marcone A, Gallone S, Serpente M, Cantoni C, Bonsi R, Cioffi S, Cappa S, Franceschi M, Rainero I, Mariani C, Scarpini E, Galimberti D (2013) Expression of the transcription factor Sp1 and its regulatory hsa-miR-29b in peripheral blood mononuclear cells from patients with Alzheimer's disease. *J Alzheimers Dis* 35, 487–494. [PubMed: 23435408]
- [22]. Yu J, Luo H, Li N, Duan X (2015) Suppression of type I collagen expression by miR-29b via PI3K, Akt, and Sp1 pathway, Part II: An in vivo investigation. *Invest Ophthalmol Vis Sci* 56, 6019–6028. [PubMed: 26393468]
- [23]. Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. [PubMed: 15685193]
- [24]. Van den Hove DL, Kompotis K, Lardenoije R, Kenis G, Mill J, Steinbusch HW, Lesch KP, Fitzsimons CP, De Strooper B, Rutten BP (2014) Epigenetically regulated microRNAs in Alzheimer's disease. *Neurobiol Aging* 35, 731–745. [PubMed: 24238656]
- [25]. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3, 1101–1108. [PubMed: 18546601]
- [26]. Nelson PT, Wang WX, Rajeev BW (2008) MicroRNAs (miRNAs) in neurodegenerative diseases. *Brain Pathol* 18, 130–138. [PubMed: 18226108]
- [27]. Saugstad JA (2010) MicroRNAs as effectors of brain function with roles in ischemia and injury, neuroprotection, and neurodegeneration. *J Cereb Blood Flow Metab* 30, 1564–1576. [PubMed: 20606686]
- [28]. Macfarlane LA, Murphy PR (2010) MicroRNA: Biogenesis, function and role in cancer. *Curr Genomics* 11, 537–561. [PubMed: 21532838]
- [29]. Launianen T, Sajantila A, Rasanen I, Vuori E, Ojanpera I (2010) Adverse interaction of warfarin and paracetamol: Evidence from a post-mortem study. *Eur J Clin Pharmacol* 66, 97–103. [PubMed: 19779704]
- [30]. Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q, Rigoutsos I, Nelson PT (2008) The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. *J Neurosci* 28, 1213–1223. [PubMed: 18234899]

- [31]. Wang H, Liu J, Zong Y, Xu Y, Deng W, Zhu H, Liu Y, Ma C, Huang L, Zhang L, Qin C (2010) miR-106b aberrantly expressed in a double transgenic mouse model for Alzheimer's disease targets TGF-beta type II receptor. *Brain Res* 1357, 166–174. [PubMed: 20709030]
- [32]. Schonrock N, Ke YD, Humphreys D, Staufenbiel M, Ittner LM, Preiss T, Gotz J (2010) Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. *PLoS One* 5, e11070. [PubMed: 20552018]
- [33]. Lausted C, Lee I, Zhou Y, Qin S, Sung J, Price ND, Hood L, Wang K (2014) Systems approach to neurodegenerative disease biomarker discovery. *Annu Rev Pharmacol Toxicol* 54, 457–481. [PubMed: 24160693]
- [34]. Doubi-Kadmiri S, Benoit C, Benigni X, Beaumont G, Vacher CM, Taouis M, Baroin-Tourancheau A, Amar L (2016) Substantial and robust changes in microRNA transcriptome support postnatal development of the hypothalamus in rat. *Sci Rep* 6, 24896. [PubMed: 27118433]
- [35]. Heicklen-Klein A, Ginzburg I (2000) Tau promoter confers neuronal specificity and binds Sp1 and AP-2. *J Neurochem* 75, 1408–1418. [PubMed: 10987820]
- [36]. Citron BA, Dennis JS, Zeitlin RS, Echeverria V (2008) Transcription factor Sp1 dysregulation in Alzheimer's disease. *J Neurosci Res* 86, 2499–2504. [PubMed: 18449948]
- [37]. Docagne F, Gabriel C, Lebeurrier N, Lesne S, Hommet Y, Plawinski L, Mackenzie ET, Vivien D (2004) Sp1 and Smad transcription factors co-operate to mediate TGF-beta-dependent activation of amyloid-beta precursor protein gene transcription. *Biochem J* 383, 393–399. [PubMed: 15242331]
- [38]. Bihaqi SW, Bahmani A, Subaiea GM, Zawia NH (2014) Infantile exposure to lead and late-age cognitive decline: Relevance to AD. *Alzheimers Dement* 10, 187–195. [PubMed: 23867794]
- [39]. Kang SW, Kim SJ, Kim MS (2017) Oxidative stress with tau hyperphosphorylation in memory impaired 1,2-diacetylbenzene-treated mice. *Toxicol Lett* 279, 53–59. [PubMed: 28734998]
- [40]. Dosunmu R, Alashwal H, Zawia NH (2012) Genome-wide expression and methylation profiling in the aged rodent brain due to early-life Pb exposure and its relevance to aging. *Mech Ageing Dev* 133, 435–443. [PubMed: 22613225]
- [41]. Alashwal H, Dosunmu R, Zawia NH (2012) Integration of genome-wide expression and methylation data: Relevance to aging and Alzheimer's disease. *Neurotoxicology* 33, 1450–1453. [PubMed: 22743688]

Fig. 1.

Correspondence between *APP* mRNA, miRNA, and protein levels at PND20 and 50 in control and Pb-exposed hTau transgenic mice. (a) mRNA levels; (b) miR-106b amount relative to sno202; (c and d) change in protein expression levels and quantification by normalization to GAPDH, control animals (dashed line) and Pb-E animals (solid lines); (e) summary of outcomes: Up or down arrows denote increase or decrease in levels (PND20 control compared to control PND50; PND20 Pb to PND50 Pb), Arrows colored green denote an expected outcome in the protein levels relative to miRNA levels. Results are expressed as mean $\pm$ S.E.M using the ANOVA followed by Newman-Keuls *post hoc* test ($p < 0.05$, $p < 0.01$ considered significant compared to control), $n = 3$ and measurements were in triplicates.

Fig. 2.

Correspondence between *SP1* mRNA, miRNA, and protein levels at PND20 and 50 in control and Pb-exposed hTau transgenic mice. (a) mRNA levels; (b) miR-124 amount relative to sno202; (c and d) change in protein expression levels and quantification by normalization to GAPDH, control animals (dashed line) and Pb-E animals (solid lines); (e) Summary of outcomes: Up or down arrows denote increase or decrease in levels (PND20 control compared to control PND50; PND20 Pb to PND50 Pb). Arrows colored green denote an expected outcome in the protein levels relative to miRNA levels. Results are expressed as mean $\pm$ S.E.M using the ANOVA followed by Newman-Keuls *post hoc* test ($p < 0.05$, $p < 0.01$ considered significant compared to control), $n = 3$ and measurements were in triplicates.

Fig. 3.

DNMT1/mRNA, miRNA, and protein levels at PND20 and 50 in control and Pb-exposed hTau transgenic mice. (a) mRNA levels; (b) miR-148a amount relative to sno202; (c and d) change in protein expression levels and quantification by normalization to GAPDH, control animals (dashed line) and Pb-E animals (solid lines); (e) Summary of outcomes: Up or down arrows denote increase or decrease in levels (PND20 control compared to control PND50; PND20 Pb to PND50 Pb), horizontal arrows denote no change. Arrows colored green denote an expected outcome in the protein levels relative to miRNA levels. Results are expressed as mean $\pm$ S.E.M using the ANOVA followed by Newman-Keuls *post hoc* test ($p < 0.05$, $p < 0.01$ considered significant compared to control), $n = 3$ and measurements were in triplicates.

Fig. 4. *DNMT3a* mRNA, miRNA, and protein levels at PND20 and 50 in control and Pb-exposed hTau transgenic mice. (a) mRNA levels; (b) miR-29b amount relative to sno202; (c and d) change in protein expression levels and quantification by normalization to GAPDH, control animals (dashed line) and Pb-E animals (solid lines); (e) Summary of outcomes: Up or down arrows denote increase or decrease in levels (PND20 control compared to control PND50; PND20 Pb to PND50 Pb), horizontal arrows denote no change. Arrows colored green denote an expected outcome in the protein levels relative to miRNA levels. Results are expressed as mean $\pm$ S.E.M using the ANOVA followed by Newman-Keuls *post hoc* test ($p < 0.05$, $p < 0.01$ considered significant compared to control), $n = 3$ and measurements were in triplicates.

Fig. 5. *MeCP2* mRNA, miRNA, and protein levels at PND20 and 50 in control and Pb-exposed hTau transgenic mice. (a) mRNA levels; (b) miR-132 amount relative to sno202; (c and d) change in protein expression levels and quantification by normalization to GAPDH, control animals (dashed line) and Pb-E animals (solid lines); (e) Summary of outcomes: Up or down arrows denote increase or decrease in levels (PND20 control compared to control PND50; PND20 Pb to PND50 Pb). Arrows colored green denote an expected outcome in the protein levels relative to miRNA levels. Results are expressed as mean $\pm$ S.E.M using the ANOVA followed by Newman-Keuls *post hoc* test ($p < 0.05$, $p < 0.01$ considered significant compared to control), $n = 3$ and measurements were in triplicates.

Table 1

Forward and reverse primers used in qPCR assay

Target	FORWARD PRIMER	REVERSE PRIMER
<i>SP1</i>	5'-TCATACCAAGGTGCAAAACCAA-3'	5'-AGG TGA TGT TCC CAT TCA GG-3'
<i>APP</i>	5'-GGTTCTGGGCTGACAAACAT-3'	5'-GTG ATG ACA ATC ACG GTT GC -3'
<i>MeCP2</i>	5'-CAGCAGCATCTGCAAGAAG-3'	5'-TCC ACA GGC TCC TCT CTG TT-3'
<i>DNMT1</i>	5'-GAGTCTTCGACGTCACACA-3'	5'-AGC TAC CTG CTC TGG CTC TG-3'
<i>DNMT3a</i>	5'-CTTGGAGAAGCGGAGTGAAC-3'	5'-GGA TTC GAT GTT GGT GTC CT-3'
<i>GAPDH</i>	5'-AGGTCGGTGTGAACGGAT TTG-3'	5'-TGTAGACCATGTAGTTGAGGT CA-3'
miR-29b	[miRBaseID: MI0000143]: GCTGGTTTCATATGGTGTTTA	Universal primer provided with the kit
miR-106b	[miRBase ID: MI0000407]: TAAAGTGTGACAGTGCAGAT	Universal primer provided with the kit
miR-124	[miRBase ID: MI0000716]: CGTGTTCACAGCGGACCTTGAT	Universal primer provided with the kit
miR-132	[miRBase ID: MI0000158]: AACCGTGGCTTTCGATTGTTAC	Universal primer provided with the kit
miR-148	[miRBase ID: MI0000550]: AAAGTTCTGAGACACTCCGACT	Universal primer provided with the kit
<i>Sno202</i>	5'-GCTGTACTGACTTGATGAAG-3'	5'-CATCAGATGGAAAGGGTTTC-3'