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Single-cell investigation of lead toxicity from neurodevelopment to neurodegeneration: Current review and future opportunities

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Abstract

Human exposure to the metal lead (Pb) is prevalent and associated with adverse neurodevelopmental and neurodegenerative outcomes. Pb disrupts normal brain function by inducing oxidative stress and neuroinflammation, altering cellular metabolism, and displacing essential metals. Prior studies on the molecular impacts of Pb have examined bulk tissues, which collapse information across all cell types, or in targeted cells, which are limited to cell autonomous effects. These approaches are unable to represent the complete biological implications of Pb exposure because the brain is a cooperative network of highly heterogeneous cells, with cellular diversity and proportions shifting throughout development, by brain region, and with disease. New technologies are necessary to investigate whether Pb and other environmental exposures alter cell composition in the brain and whether they cause molecular changes in a cell-type-specific manner. Cutting-edge, single-cell approaches now enable research resolving cell-type-specific effects from bulk tissues. This article reviews existing Pb neurotoxicology studies with genome-wide molecular signatures and provides a path forward for the field to implement single-cell approaches with practical recommendations.

Keywords

Lead (Pb); Brain; Neurotoxicology; Single-cell transcriptomics; Omics

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Introduction

Lead (Pb) exposure continues to represent a substantial public health burden, with more than 1.8 million children in the US estimated to have blood Pb levels above 3.5 μg/dL [1]. Despite policy changes aimed to reduce environmental release of Pb over the last several decades, Pb is persistent in the environment. Ongoing sources of Pb include housing and infrastructure built prior to 1970 and contamination from waste recycling, mining, and smelting industries. The developing brain is the most susceptible to Pb and exposure can lead to life-long changes in brain function. Pb crosses the placental barrier and can also be passed from a pregnant person to an infant via breast milk [2,3]. Infants can also ingest Pb from contaminated drinking water, formula, or foods [4]. Infants and children commonly engage in hand-to-mouth behaviors as they interact with their environment and have higher rates of respiration relative to adults, facilitating ingested and inhaled exposures. Pb is more efficiently absorbed by the gut during periods of rapid development (e.g., infancy and childhood) or periods of bone mobilization (e.g., pregnancy and osteoporosis), and it readily crosses the blood–brain barrier. Developmental Pb exposure is associated with numerous adverse neurocognitive outcomes, including impaired learning and memory formation [5]. In adults, Pb exposure is associated with cognition cross-sectionally and rate of cognitive decline [6]. Pb exposure is ubiquitous and associated with adverse neurological outcomes, thus investigating the molecular and cellular basis of Pb toxicity is essential for both treatment and advocating for further preventative measures to protect vulnerable populations.

Pb induces oxidative stress and disrupts cellular homeostasis of essential metals such as calcium [7–9]. These molecular effects of Pb occur at all stages of development and into adulthood, but other toxic effects may be specific to a developmental stage or disease state. Throughout neurodevelopment specifically, multiple physiological parameters rapidly change as cells differentiate. Exposures *in utero* may have substantially different effects on the brain than those that occur during early childhood and adolescence, given the dramatic brain maturation between these periods [10]. Indeed, broad uniform changes are often seen following early developmental exposures, when stem and progenitor cell populations are abundant (Figure 1(a)). When exposures occur later, clustered responses become the norm as cellular populations have taken on distinct characteristics and often exhibit population-specific responses to environmental challenges (Figure 1(b)). One powerful method for monitoring the impacts of Pb exposure at various time points is the application of varying omics platforms, which includes assessing genome-wide impacts on epigenomics and transcriptomics. For example, single-cell sequencing allows users to cluster cells with similar gene expression profiles and identify specific cell types (Figure 1(c)). These experiments can be performed across varying developmental and disease stages to reveal stage- and cell type-specific effects of Pb exposure.

In this article, we review the current state of the literature on Pb exposure's molecular omic effects on brain tissues. Existing studies using bulk brain tissue and sorted or cultured cells were critical first steps for the field, which is ready to apply emerging technologies to simultaneously investigate effects within and across cell types. We describe these cutting-edge single-cell and single-nuclei approaches, and share opportunities for their application

to Pb neurotoxicology. We provide the rationale and practical workflows for investigating the cell type-specific impacts of Pb exposure on neurodevelopment and neurodegeneration.

Pb exposure causes molecular changes in bulk brain tissues and sorted cells

Pb neurotoxicology studies generally focus on molecular impacts derived from bulk analysis of whole brain or specific brain regions. This approach is common for practical reasons of tissue size, and for scientific rationale including disease relevance, such as examinations of the substantia nigra with regard to Parkinson's Disease. Pb exposure affects all brain regions including the cortex, hippocampus, and cerebellum where neuroinflammation and tau phosphorylation [11], as well as changes in gene expression and epigenetic regulation, have been observed. Developmental Pb exposure has been associated with increased expression of beta amyloid protein 40 (Aβ40) [12] and metallothionine 3 (MT-3) [13], as well as differential expression of epigenetic regulators of the amyloid-β precursor (AβPP) gene [14] in cortical tissue. Additionally, differential expression of microRNAs that regulate neurodegenerative pathways has been observed in the hippocampus [15], as well as with those involved in oxidative stress in the cerebellum [16]. Recent studies have built on the successes of targeted pathway analyses and moved towards unbiased, genome-wide discovery analyses.

A summary of various bulk 'omics' studies assessing changes in the brain following developmental Pb exposure from the last 10 years is included in Table 1. These important studies demonstrate that early life exposure to Pb has observable gene expression and DNA methylation/hydroxymethylation signatures in bulk brain tissues (cortex, hippocampus, and whole brain). Importantly, each of these studies exhibit a high degree of heterogeneity with respect to exposure dose, timing, model organism, brain region, and omic platform, which poses a challenge for replication testing and comparisons across studies. This is important to consider as Pb effects may vary over the time course and duration of exposures. For example, metallothioneins are induced by acute exposure, but are not likely to be a persistent signal [17]. Further, bulk tissue studies represent an average signal across all cells present in the tissue [18], and an important next step is to examine whether these average changes reflect a Pb exposure molecular response across all cell types, a molecular response in a particular vulnerable cell type, or a change in cell proportions.

While bulk tissue analyses provide an important initial impression of how Pb exposure contributes to dysregulation and disease risk in the brain, there is a need for further assessment at the cellular level, because Pb is likely to induce cell type-specific effects. For example, Pb is known to alter cellular metabolism by inhibiting mitochondrial electron transport chain complexes [30] and Pb's metabolic effects are likely cell type-specific given that glia are highly glycolytic at baseline and neurons rely almost exclusively on oxidative phosphorylation [31]. It would be challenging to study these effects in pure cultures of neurons or glia because these cells engage in cooperative metabolism [31]. Figure 2 shows how bulk tissue measurements are unable to resolve cell-specific effects, where direct effects on two or more cell types are indistinguishable from a larger change in a singular cell type.

Exceptionally few studies have examined the cell type-specific effects of Pb exposure in the brain. Antibody separation of cortical neural nuclei exposed to Pb exhibited hypomethylation of genes associated with neurodevelopment and cognition [23]. While this approach provides a perspective on how Pb perturbs epigenetic regulation in neurons specifically, it precludes an understanding of how other cell types in the same tissue are affected by the same exposure. Cell purification strategies and *in vitro* platforms demonstrate specific cell types do respond to Pb in distinct ways, but they require an a priori hypothesis about which cell types to investigate. New technologies with single-cell resolution make it possible to investigate multiple cell types from bulk brain tissues simultaneously.

Single-cell approaches to study neurologic disorders

The brain is composed of many cell types that are transcriptionally and functionally diverse (Figure 1(a,b)). Bulk RNA-sequencing analyses reflect composite changes in gene expression and are unable to differentiate changes in cell type proportion, uniform changes in gene expression, or cell type-specific changes contributing to the observed trends (Figure 2) [18]. Recent methodological advances allow the resolution of molecular signals at the level of individual cells [32]. These types of single-cell or single-nuclei approaches will enable toxicologists to compare how individual cells respond to toxicants as well as to detect heterogeneous responses within a single-cell type. For example, astrocytes can adopt an inflammatory, reactive state called astrogliosis in response to chronic Pb exposure [33,34] in the hippocampus, but not cerebellum [33], suggesting a possible heterogeneous response. Single-cell sequencing has shown that astrogliosis can occur in a small subset of astrocytes [35] and this approach could be useful for examining Pb-induced reactivity. Vulnerable or reactive cell types can be identified by implementing a single-cell sequencing approach for toxicology studies.

Single-cell sequencing typically first requires dissociating 3D tissue (or 2D cultures) into single cells or nuclei using enzymatic digestion and/or mechanical perturbation. The dissociation approach should be compatible with the goals of the experiment. For brain tissue specifically, dissociation kits such as the Neural Tissue Dissociation Kits (Miltenyi Biotech) are designed to stabilize neurons and prevent neuronal signaling during dissociation. The use of RNAse inhibitors will improve RNA quality during tissue dissociation and processing. Additionally, transcription and translation inhibitors limit the artifacts from *ex vivo* gene expression profiles that are induced by enzymatic dissociation approaches [36], which seems to be most prevalent in dynamic cell types like microglia. When dissociating nervous tissue, it is important to include compounds to stabilize the gene expression signals.

Traditional dissociation approaches for single-cell analyses separate whole cells, which allows for analysis of total cellular RNA levels, containing potentially long-term cytoplasmic RNA. Some cell types are more vulnerable to loss during dissociation due to their size and shape (e.g., neurons). If the cell population of interest is a vulnerable or relatively rare cell type, the cell suspension can be enriched using fluorescence-assisted cell sorting (FACS) [37] or similar approaches. An alternative to whole-cell separation is

to isolate nuclei. Vulnerable cells, such as neurons, often retain better integrity in nuclei isolation and can be captured in greater numbers without enrichment. Nuclei dissociated from frozen tissue are not subject to dissociation-associated *ex vivo* gene expression artifacts. An important tradeoff to note, nuclei isolation restricts downstream analyses to nuclear RNA levels, which reflect a snapshot of current transcription. Based on the research question of interest, dissociation choices (cell versus nuclei, and any potential enrichment) require careful consideration in study design and will impact the types of information (total RNA versus nuclear RNA) that is captured in sequencing.

Following dissociation, samples can proceed directly to the next step. In a common commercial single-cell platform, 8 samples are processed simultaneously. If all samples (or a balanced mixture of exposed and control samples) are not available at the same time, this may result in batch effects. Often, intermediate storage is required to aggregate samples and limit batch effects. In this case, dissociated cells or nuclei can be archived frozen or fixed, such as with the Single Cell Gene Expression Flex Kit (10X Genomics). Taking care to effectively store samples can ensure samples are balanced across runs and single-cell measures are not confounded by batch.

Many single-cell approaches use microfluidics to dilute single cells into droplets, where RNA transcripts are barcoded before library preparation [32]. This approach was originally termed drop-seq and is now available from commercial platforms including Chromium (10X Genomics). Typically, each RNA transcript is barcoded with a unique molecular identifier that is associated with each individual droplet, assumed to be one single cell (e.g., barcode for 10x Genomics) based on dilution calculations. In many cases, the polyA tail is used to isolate RNA transcripts and only the 3′ portion of the transcript is ultimately sequenced. Alternatively, cells can be physically isolated in microwells on a processing plate, which allows for deeper and full transcript sequencing, but is more labor-intensive and has lower throughput. In either case, RNA-derived cDNA is then amplified and indexed with standard index and priming sequences compatible with the downstream sequencing platform (e.g., i5 or i7 indices for Illumina sequencers).

Once a cDNA library is constructed, investigators will decide on approaches for sequencing. While only shallow sequencing is necessary for cell class identification, 20–50 k reads per cell is likely necessary to detect cell class subtypes [38,39] and subtle transcriptomic responses (e.g., at time points post-exposure) may require deeper sequencing. Even when high sequencing saturation is achieved, single-cell RNA-seq is much shallower than bulk sequencing, where it is routine to sequence 20+ million reads per sample. Sequences from individual cells can be pooled together in ‘pseudobulk’ analysis and may approach bulk sequencing read numbers. Most single-cell library preparation chemistries are plagued by low-sampling artifacts including a strong bias toward abundant transcripts and inefficient capture of rare transcripts [32]. Advances in sequencing technologies, such as 26 billion possible reads per flow cell with the Illumina NovaSeq X series, can provide the opportunity to increase the depth of sequencing per cell.

After sequencing, reads are filtered for quality control, aligned to the genome, and cells are demultiplexed. Next, data sets are further filtered to remove low-quality cells: 1) dying cells

with high mitochondrial RNA or low number of reads, and 2) multiplets with high read counts. The data are then normalized. When identifying informative features in the data, genes with low variability can be excluded. Dimension reduction approaches find structure within the data and these are typically visualized in two dimensions on a Uniform Manifold Approximation and Projection (UMAP) plot (Figure 1(c)). The largest source of variability in gene expression is typically driven by cell identity. Cells can be assigned to clusters based on similarity in expression profiles. Comparing each cluster to all others using differential expression analysis generates a list of marker genes that correspond to cell cluster identity. These are commonly visualized on a heatmap (Figure 1(c)), dot plot, or violin plot. There are several automated approaches for annotating cell clusters [40], but manual assignment is typically required using resources such as those in Table 2. Cell-specific marker genes vary by developmental stage and species (Table 2). Dimension reduction approaches are valuable for visualizing complex data sets containing thousands of genes across thousands of cells. However, all dimensionality reduction and clustering approaches have inherent statistical biases and by reducing complexity, distort the underlying data [41]. This is an active area of research and investigators should stay current with best practices.

After cluster annotation, the downstream analysis strategy is determined by researcher priorities. Investigators can test for differences in proportions of cell clusters by exposure using beta regression. Developmental trajectories from progenitor cells to more differentiated cells can be inferred using bioinformatic approaches where users define a starting cluster and place the remaining cells on a continuous path termed ‘pseudotime’ [42]. This is a particularly helpful approach if the exposure or disease of interest is expected to alter cell differentiation. Additionally, one can examine overall differences in expression at each gene (accounting for cell composition) and generate a list of differentially expressed genes (DEGs) in the tissue. Similarly, within each cell cluster investigators can test for DEGs specific to that cell type. The magnitude of effect and level of statistical significance across DEGs can be visualized on a volcano plot. DEGs are then used as input into gene ontology (GO) and gene set enrichment analysis (GSEA) [42]. Many follow-up analyses can be considered based on the specific scenario.

Application of single-cell analyses to the brain and in Pb neurotoxicology

Single-cell approaches are becoming increasingly accessible to new users due to the development of commercialized kit-based pipelines and decreasing sequencing cost. The neuroscience field is particularly well positioned to utilize this technology because of the diversity of cell types in the brain (Figure 1(a,b)). Cellular heterogeneity is especially interesting during development when transient progenitor populations arise and differentiate. A single gestational week in human brain development can correspond to a large change in cellular composition as shown in large single-cell sequencing studies of the fetal brain with samples spanning 5–41 weeks of gestation [48,49]. These studies have identified new progenitor cell populations including subclasses of radial glia [48] and multipotent glial progenitors [49].

High cellular heterogeneity in the brain underscores the need for developmentally and tissue-appropriate cluster annotation datasets (Table 2) and suggests that toxicity studies

during these windows should be cognizant of cell differentiation. Pseudotime analyses may be an effective approach to identify cell differentiation trajectory differences by Pb exposure, considering developmental timing and duration of exposure. Pb is a known neurodevelopmental toxicant, and these approaches may be particularly relevant for examining critical periods of increased vulnerability in development and changes in cell fate.

Single-cell or nuclei sequencing can be leveraged to quantify changes in tissue composition based on the proportion of each cell type captured from tissue with and without perturbation by toxicant exposure or disease. In the aging brain, different cell types may be more or less sensitive to disease or toxicological insults, such as dopamine neurons which are more sensitive to ROS-inducing rotenone than other neurons due to their higher mitochondrial metabolism [54]. The reliance on single-cell RNA-seq alone to quantify tissue composition and proportion of different cell types is not advisable because particular cells may be more vulnerable to loss during cell dissociation. Single-nuclei approaches are more likely to retain relative proportions of cells and may be better suited for examining changes in relative proportions of different cell types.

Although Pb is associated with developmental toxicity, exposures are also associated with an increased risk of neurodegenerative diseases including Alzheimer's disease (AD) [50] and Parkinson's Disease (PD) [51]. The cell type-specific effects of Pb exposure have yet to be extensively evaluated with regard to neurodegenerative outcomes, though single-cell analyses of ND progression have laid the foundation for such work in the future. The Seattle Alzheimer's Disease Brain Cell Atlas (SEA-AD.org) is an open-access resource covering the full spectrum of AD severity and progression. It includes single-nuclei RNA-seq, ATAC-seq, quantitative pathology, spatial MERFISH, and clinical disease severity measurements. These data are easily visualized on the SEA-AD Allen Brain Atlas website. The earliest analysis of this new data set demonstrated the relative abundance of each cell class alongside the pseudoprogression score to show different cells lost and generated over the course of AD [53].

Similarly, large clinical data sets from the Religious Orders Study and Rush Memory and Aging Project (ROSMAP) have combined single-nuclei approaches with disease progression metadata. One of the earliest analyses of the ROSMAP AD single-nucleotide data set reported large transcriptomic changes with cell-specificity early in disease, before more severe pathology arose, and more uniform transcriptomic changes later in the disease [52]. Follow-up work with the expanded ROSMAP data set (2.3 million nuclei, 427 donors) compared gene expression across groups with increasing severity of AD pathology, which was marked by a loss of somatostatin inhibitory neuron subtypes. These subtypes were not lost in donors who presented AD pathology and also atypical resilience to cognitive impairment [53]. Early AD has also been characterized by changes in excitatory neurons including β-amyloid production [54], and loss of genetic stability marked by double-strand breaks and enrichment of somatic mosaic gene fusions [55]. Sn-ATAC-seq revealed differential changes in accessibility in neurons (early AD) and glia (late AD), and a global loss of epigenome integrity leading to loss of cell identity later in disease [56]. Combining

sn-RNAseq and sn-ATAC-seq enabled Sun et al. to identify 12 distinct microglia states as the AD progressed and to identify the transcription factors that mediated these states [57].

Single-cell sequencing is well positioned to identify different cell types, and can therefore be leveraged to quantify changes in tissue cmoposition based on the proportion of each cell type captured from tissue with and without exposure. In the brain, different cell types may be more or less sensitive to toxicologic insults or disease. For example, dopamine neurons are more sensitive to reactive oxygen stress-inducing rotenone than other neurons due to their higher mitochondrial metabolism [58] and these cell proportion changes may be detected with single-cell RNA-seq. Single-cell RNA-seq based tissue composition and cell type proportion should be validated using pathology, as particular cells may be more vulnerable to loss during cell dissociation. Single-nuclei approaches may be more likely to retain relative proportions of cells and may be better suited for examining changes in relative proportions of different cell types.

Developmental exposures that alter cell fate may also change the proportion of captured cells. Zhang et al. found that manganese exposure is associated with an increase in GABAergic neurons and undefined neurons, and a loss of dopaminergic and cholinergic neurons in the zebrafish brain using single-cell RNA-seq. They used pseudotime analysis to map exposures (control, low, high) onto different cell populations and show a change in differentiation trajectory [59]. Likewise, single-cell analyses have been used to show changes in cell proportions following exposure to methylmercury (MeHg) [60,61]. Loan et al. exposed mice to early life (embryonic day 0–15) MeHg and found an increase in autism spectrum disorder-like behaviors in adulthood. Using single-cell RNA-seq and RNA velocity analysis, they found that MeHg exposure increases neuronal differentiation from fetal radial glial precursors at embryonic day 13.5 after two weeks of exposure [60].

To date, one study has employed single-cell analyses in Pb neurotoxicology [29]. Mouse dams were exposed to Pb via the drinking water 2 weeks before mating and through weaning. Single-cell RNA-seq was performed on hippocampal brain tissue from 4 mice (2 males and 2 females) exposed to Pb *in utero* and 4 non-exposed controls (2 males and 2 females) at 5 months of age. The transcriptomic changes observed at 5 months are thus persistent changes to cell identity and function, not an acute response to Pb. Overall, glial cells exhibited the most transcriptomic changes associated with Pb. The single-cell approach quantified a 12.4% increase in oligodendrocytes in Pb-exposed animals. To test whether this was due to a change in oligodendrocyte differentiation, they re-analyzed data from *in vitro* neural progenitor cell cultures exposed Pb-acetate or control conditions [62] and measured early Pb-associated increases in oligodendrocyte marker genes including *OLIG1, OLIG2, PDGFRA*, and *MBP*. Bulk analysis of this tissue would likely not have revealed the changes in cell type proportions and may have been unable to detect the decrease in oligodendrocyte gene expression. This further promotes the idea that single-cell analyses are an important next step in our assessment of how neurodevelopmental exposures impact various regions of the brain and what cell-specific responses may mean for disease risk. Future work can build on this by examining the functional or behavioral outcomes related to this increase in oligodendrocytes.

Recommendations and summary

Pb's effects on the brain are complex and likely vary by cell type, developmental time period of exposure, and outcome measurement. Recent advances in single-cell approaches make the investigation of cell-type-specific effects feasible. The first (and currently only) single-cell RNA-seq study of Pb exposure in the brain was published in 2020 [29]. In this study, the majority of the cells captured were non-neuronal: endothelial cells (27%), microglia (27%) and oligodendrocytes (15%). These abundances do not reflect *in vivo* tissue proportions and highlight the unequal vulnerabilities of different cells to dissociation. In particular, neurons are not well preserved during dissociation of whole cells, but they are better preserved as nuclei. In this dataset, the cell classes with the most differentially expressed genes were also those with the most cells and statistical power. For this reason, additional studies are necessary to better characterize the long-term effects of early-life Pb on neurons and more rare cell types.

As with any other experiment, single-cell experiments should include at least 3 biological replicates (i.e., individual animals) and often more in cases of high heterogeneity such as patient-derived samples. More complex tissues with more cell types require more cells sequenced so that each cell type has a sufficient number of cells for comparing exposed vs. unexposed groups. In some cases, it is better to focus on a single brain region to ensure adequate coverage of each cell type. For example, a comparison of 1000 dopaminergic neurons comprised of 500 control versus 500 exposed cells each sequenced to a depth of 20,000 reads per cell provides only 10 million reads, which is significantly lower than standard bulk approaches, which are commonly 20–40 million reads/sample. This sequencing depth is adequate for broad cell identity, but quantifying subtle transcriptomic effects of metal exposure may be hard to distinguish from noise without deeper sequencing.

One major limitation of single-cell RNA-seq is that it is biased toward more abundant transcripts and rare, but biologically meaningful, differences may never be captured even when sequencing depth is saturated. For this reason, if only a few cell types are of interest it would be more informative to purify individual cell types by FACS, immunopanning, or magnetic activated cell sorting (MACs) and perform bulk sequencing. Additionally, the most common single-cell RNA-seq chemistries are limited to the 3' portion of the transcript and these libraries cannot be used to examine changes in transcript splicing. For the reasons mentioned above, single-cell sequencing and bulk sequencing approaches provide different, and often complementary, information and both techniques have their individual caveats to consider. Careful experimental design that is tailored to the research question of interest will ensure efficient use of cells and resources.

Importantly, experimental findings from a single-cell data set should be validated using orthogonal approaches. For example, transcriptional changes can be validated at the RNA level by RNAscope or the protein level by immunohistochemistry, dot blots, or western blots. Changes in cell proportion can be validated using immunohistochemistry, flow cytometry, or proliferation and cell survival assays. There are several papers describing best practices for single-cell analysis [32], and new and existing users should consciously seek out and use new resources as they become available in this rapidly changing field.

Techniques are increasingly becoming available to conduct single-cell measures beyond gene expression, including single-cell ATAC-sequencing for chromatin accessibility. Single-cell sequencing is typically performed on a single-cell suspension with loss of spatial information, and although it is beyond the scope of this article to discuss in detail, multiple approaches now allow visualization of transcripts within tissue sections (e.g., 10x Visium, Nanostring). Of note, these approaches typically use probe sets rather than unbiased sequencing. Rapidly advancing tools will enable a detailed and specific investigation of the molecular effects of Pb on the brain.

In summary, human Pb exposure remains an ongoing health concern and is associated with neurodevelopmental and neurodegenerative disorders. Pb exposure causes widespread differences in gene expression in bulk tissue, sorted cells, and in cell culture. We propose that to resolve the cell types that underlie these bulk changes, single-cell analyses are needed. We provide recommendations for implementing single-cell approaches in Pb neurotoxicology. These new technologies represent an exciting opportunity for neurotoxicologists to advance genome-wide molecular discovery in a cell-type-specific manner. A more comprehensive understanding of how Pb exposure elicits cell and developmental stage-specific effects will support exposure prevention and therapeutic endeavors.

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Data availability

No data were used for the research described in the article.

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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

* * of outstanding interest

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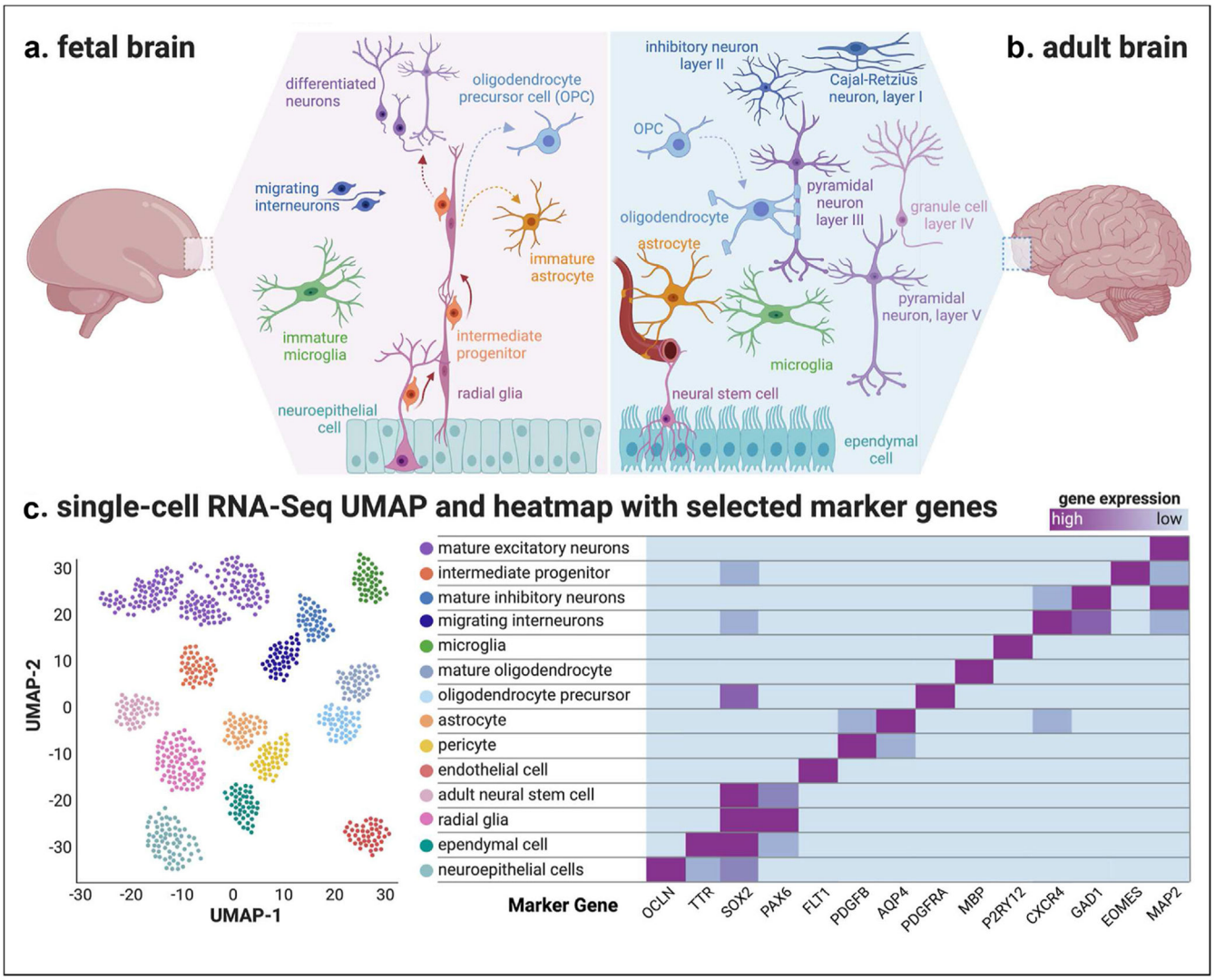

Figure 1.

Brain tissue is highly heterogeneous, containing hundreds of specialized and unique cell types. Panel (**a)** depicts a simplified cartoon of the structure and major cell types present in cortical brain tissue during fetal development. Panel (**b)** depicts the structure and major cell types present in the adult cortex. For simplicity, a small selection of cell types is shown. Panel (**c)** portrays hypothetical single-cell RNA-seq data including these brain cell types after they have been assigned to clusters and visualized in a uniform manifold approximation and projections (UMAP) plot. The heatmap shows cell-specific marker genes (x-axis) used to infer cell type for each cluster (y-axis). Relative gene expression values are for illustrative purposes only.

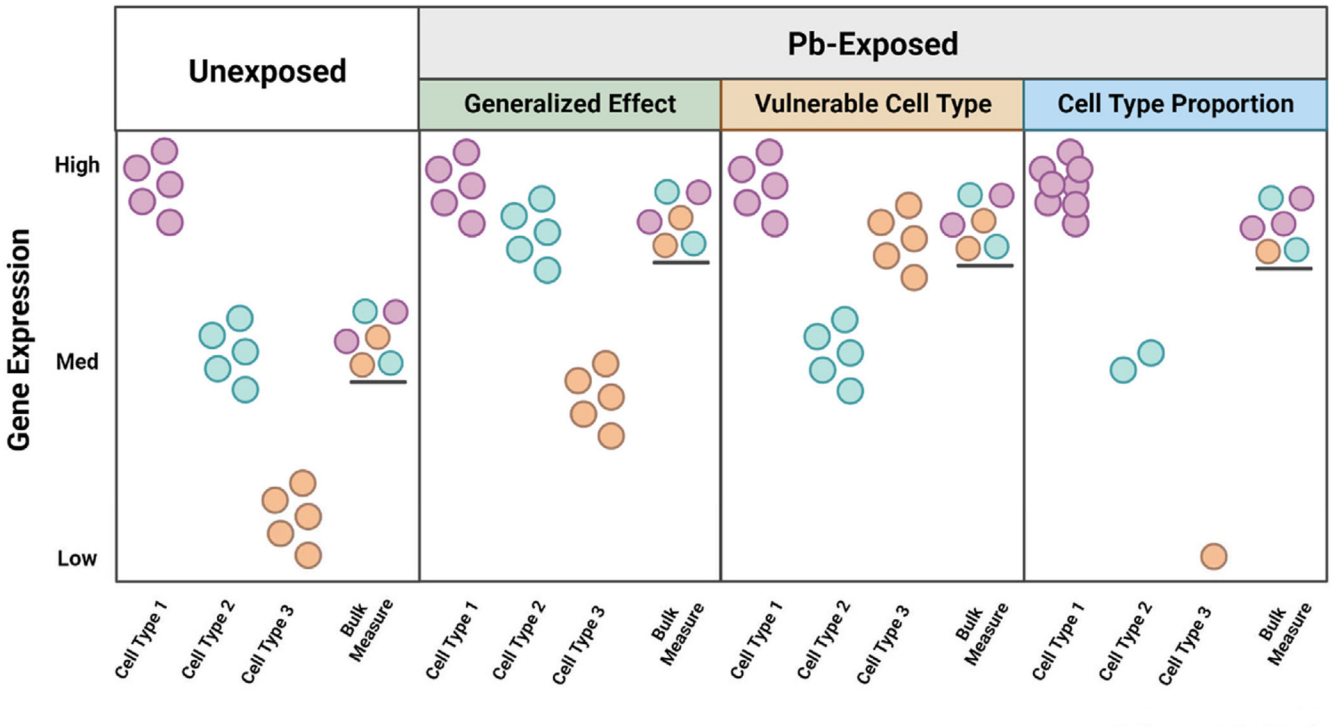

Figure 2.

Bulk tissue measures reflect mixtures of cell types present in the tissue. In this simplified example, three cell types are depicted, though many others would also be present. Pb-induced differences in gene expression in bulk tissue cannot distinguish multiple biologic scenarios, including direct effects across all cell types, strong effects in a vulnerable cell type, and shifts in cell type proportions. We showed an increase in gene expression, though an exposure could alternatively cause a decrease in gene expression. Adapted from Ref. [18].

Table 1

Summary of Pb treatment and central nervous system omics (transcriptomics and epigenomics) outcomes.

Study citation	Model	Exposure paradigm	Brain region	Omics outcome	Omics cell type	Key findings
Bulk tissue studies						
An, 2014 – [15]	Rat (Sprague Dawley)	Adult rats (F0) exposed to 100, 200, or 300 ppm via drinking water for 8 weeks.	F0 hippocampus	miRNA micro array	Mixed (bulk tissue)	7 miRNA showed differential expression in F0 rats: 6 upregulated (primarily with 300 ppm exposure), 1 downregulated (under all exposure conditions)
Sánchez-Martín, 2015 – [19]	Mouse (C57/BL6)	Adult female mice (F0) exposed to 0, 3 or 30 ppm Pb acetate via drinking water, 2 weeks prior to mating through weaning	F1 cortex, hippocampus	Methyl-seq	Mixed (bulk tissue)	Differential methylation seen in F1 males and females following gestational exposure, with DNA hypermethylation more prevalent among females.
Meyer, 2020 – [20]	Zebrafish (EK)	F0 zebrafish exposed to 10uM Pb2+ during embryonic development	F2 whole brain	Transcriptome (QuantSeq)	Mixed (bulk tissue)	648 genes differentially expressed in brains of F2 zebrafish descended from exposed F0 generation compared to control.
Wang, 2023 – [21]	Mouse (C57/BL6)	3 week old mice (F0) exposed to 100 ppm Pb via drinking water for 8 weeks	F0 cortex	miRNA sequencing	Mixed (bulk tissue)	Differential expression of 58 miRNAs observed in F0 mice. Gene targets included those involved in synaptic vesicle formation and function.
Petroff, 2023 – [22]	Mouse (wild type, non-agouti a/a)	Adult female mice (F0) exposed to 32 ppm Pb acetate via drinking water, 2 weeks prior to mating through weaning	F1 cortex	hMeDIP-seq	Mixed (bulk tissue)	No differential hydroxymethylation in F1 females; 385 regions with higher hydroxymethylation in Pb-exposed cortex in F1 males. Differential 5hmC detected in the F1 generation in genes related to neuronal and neural function according to pathway analysis.
Sorted or cultured animal cell studies						
Dou, 2019 – [23]	Mouse (wild type, non-agouti a/a)	Adult female mice (F0) exposed to 0, 2.1 or 32 ppm Pb acetate via drinking water, 2 weeks prior to mating through weaning	F1 cortex	Genome-wide promoter methylation	Cortical neuron	Largely hypomethylation at promoters identified in the F1 generation, at both low and high Pb exposure.
Gu, 2019 – [24]	Rat (Sprague Dawley)	Adult female rats (F0) exposed to 250 ppm Pb acetate via drinking water, 1 week after mating through PND60	F1 hippocampus	ChIP–chip analysis	Primary neuronal cultures	Perturbation of H3K4me3 throughout the F1 genome in cultured hippocampal neurons.
Human iPSC or ESC differentiated cultures						
Senut, 2014 – [25]	hESC	0.4–1.9 uM Pb acetate, multiple exposure paradigms during differentiation	N/A	Global DNA methylation (Illumina HumanMethylation450 BeadChip)	Neural progenitor cells (NPCs) to neurons	Significant alteration of global methylation within 24 h of exposure. Differentiating hESCs experienced the greatest number of DMCs, relative to hESCs and NPCs.
Wagner, 2017 – [26]	hESC	1 uM Pb acetate via media	N/A	RNA-seq + ChipSeq	Neural stem cells	19 genes differentially expressed, many involved in oxidative stress response.

Study citation	Model	Exposure paradigm	Brain region	Omics outcome	Omics cell type	Key findings
Wang, 2023 – [27]	hiPSC	0.5–10 uM Pb acetate via media, 7 days	N/A	Whole exome sequencing	hiPSCs, neural progenitor cells, embryoid bodies	Genetic mutations detected in all three models, many previously identified in neural disorders.
Xie, 2023 – [28]	hiPSC	15 or 50 ppb 48 h prior to differentiation	N/A	RNA-seq	Cortical neurons	Differential gene expression, with distinct profiles for each exposure. Calcium signaling, cell structure, and metabolism pathways indicated in pathway analysis.
Single-cell Studies						
Bakulski, 2020 – [29]	Mouse (wild type, non-agouti a/a)	Adult female mice (F0) exposed to 32 ppm Pb acetate via drinking water, 2 weeks prior to mating through weaning; omics performed at 5 months in the F1 generation.	F1 hippocampus	Single-cell RNA-seq (10x Chromium)	Hippocampal cells	12% increase in proportion of oligodendrocytes and an increase inprotein folding in microglia observed in hippocampi from exposed mice relative to control in the F1 generation.

Table 2

Example resources for annotating single-cell RNA sequencing clusters from brain tissue.

Resource	Species, brain region	Developmental stage	Single-cell platform	Number of cells/nuclei
Allen Brain Map [44]	Mouse cortex and hippocampus	Adolescent, 8 week	10X Chromium 3′, Smart-Seq with FACS isolated cells	1.1 million cells, 338 clusters
http://mousebrain.org [45]	Mouse Whole nervous system	Adolescent, 6–8 weeks	10x Chromium	160,796 cells, 265 clusters
Barres lab Brain RNA-seq [46,47]	Mouse and human	Mouse Postnatal day 17 for oligos Postnatal day 7 other cell types Human Fetal gestational week 17–20 Adult 8–63 years old	NA, cells purified by immunopanning and FACS	Bulk sequencing of purified neurons, oligodendrocytes, microglia, pericytes, astrocytes, endothelial cells
UCSC Genome Browser [48]	Human Cortex and medial ganglionic eminence	Fetal gestational week 5–37	Fluidigm C1	4261 cells, 48 clusters
Seattle Alzheimer's Disease Brain Cell Atlas	Human middle temporal gyrus and dorsolateral prefrontal cortex	Adult (65+ yo)	10x Chromium 3′	2.78 million nuclei, 125 clusters
Allen Brain Map [43]	Human Motor Cortex	Adult (50–60 yo)	10X Chromium 3′	76,533 nuclei, 127 clusters